Transfer of lipophilic drugs between liposomal membranes by using the ion-exchange micro-column technique and the fluorescence dequenching effect

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You cannot teach a man anything;
you can only help him find it within himself

Galileo Galilei (1564-1642)

To my parents, Zhu Qi and Wang Ping,

y mi Hanghang

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Abstract

The parenteral and oral administration of lipophilic drugs is often problematic because of their low water solubility. Liposomes are composed of relatively biocompatible and biodegradable materials consisting of an aqueous volume entrapped by one or more bilayers of natural and/or synthetic lipids. Therefore, drug-containing liposomal formulations are considered as one of the most promising nano-particle based technologies for drug delivery to solid tumours, sites of inflammation and skin permeation. An understanding of the mechanisms involved in the transport of drug from tissues to plasma, and its subsequent transfer to the liver for degradation, is of great importance. It is necessary to develop an *in vitro* model to mimic the *in vivo* transfer of the drug in order to explain the transport mechanisms involved. The present study aims to investigate the factors which influence the transfer of lipophilic drugs and to look for the mechanisms during the drug transfer process.

Two *in vitro* models have been selected in the investigation. The first model is based on the ion-exchange micro-columns. The transfer kinetics of three compounds, paclitaxel, cholesterol and cholesteryl-oleoyl-ether has been investigated. The drugs are transferred from positively (or negatively) charged, unilamellar DOPC (1,2-Dioleoyl-sn-Glycero-3-phosphocholine) donor vesicles to 5 times excess of neutral POPC (1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine) acceptor vesicles. The vesicles were incubated in the absence of protein and were stable from fusion over the course of the experiment. At different periods of time, donor and acceptor vesicles are separated by passing throughout a column filled with CM Sepharose FF (or DEAE-Sepharose). The transfer of the drugs is then measured by HPLC or LSC.

A nearly instantaneous transfer of paclitaxel with a time constant of 0.35 minutes was obtained. The fast transfer kinetics of paclitaxel might prefer the "Collision complex model" than the "Aqueous diffusion model". However, the transfer of cholesterol shows a much slower transfer rate. A time constant of 58 minutes from negative cholesterol-containing liposomes and a time constant of 114 minutes from positive cholesterol-containing liposomes were observed. The incorporation of DOTAP in positive liposomes changed the cholesterol transfer behaviour to a two-phase transfer process. The transfer of cholesterol might prefer the "Aqueous diffusion model". The time constant obtained in transfer of COE was 353 days for positively charged liposomes, and 485 days for negatively charged liposomes. The molecular structure of COE has more similarity with the lipid molecular, thus the molecule has a strong affinity with the lipid membranes.

The second model is based on the fluorescence character of the drug temoporfin. Temoporfin shows a self-quenching effect of the fluorescence at even very low concentration. The transfer of temoporfin from donor to acceptor liposomes can be easily measured by the increase of fluorescence intensity at different time points in the *in vitro* drug transfer experiment. The transfer of temoporfin from eight formulations (four DPPC/DPPE formulations and four DSPC/DSPE formulations) was investigated. It has been proved that a higher temperature facilitates the transfer of temoporfin between liposomal membranes due to a faster molecular movement and a higher lipid fluidity at a higher temperature. Moreover, the incorporation of cholesterol facilitated the transfer of temoporfin both at the lower and higher temperatures. PEG modified formulations have a faster transfer rate than the conventional formulations only at a low temperature.

Zusammenfassung

Die parenterale und orale Verabreichung von lipophilen Arzneistoffen ist aufgrund ihrer geringen Wasserlöslichkeit häufig problematisch. Liposomen bestehen aus relativ biologisch verträglichen und bioabbaubaren Materialien: Ein wässriger Innenraum wird von einer oder mehreren Doppelschichten umschlossen, welche aus natürlichen und/oder synthetischen Lipiden zusammengesetzt sind. Aus diesem Grund stellen Wirkstoff-beladene liposomale Formulierungen eine der vielversprechendsten Nanopartikeltechnologien für den Arzneistoff-Transport und die Arzneistoff-Freisetzung in der Tumortherapie, in entzündeten Geweben und bei der Hautpenetration dar. Ein Verständnis der Mechanismen, die der Aufnahme des Wirkstoffs aus den Geweben in das Plasma und seiner anschließenden Elimination durch die Leber zugrundeliegen, ist von großer Bedeutung. Um die betroffenen Transportmechanismen zu erklären, ist es notwendig, ein In-vitro-Modell zu entwickeln, das den in-vivo Transfer des Wirkstoffs nachahmt. Das Ziel der vorliegenden Arbeit ist, die Faktoren zu erforschen, die den Transfer der lipophilen Arzneistoffen beeinflussen, und die zugrundeliegenden Mechanismen zu untersuchen.

Zwei In-vitro-Modelle wurden für die Untersuchung ausgewählt. Das erste Modell basiert auf Ionenaustausch-Mikrosäulen. Die Kinetik von drei Stoffen, Paclitaxel, Cholesterin und Cholesteryl-oleoyl-ether (COE), wurde untersucht. Die Wirkstoffe werden von positiv (oder negativ) geladenen, unilamellaren DOPC (1,2-Dioleoyl-sn-Glycero-3-phosphocholine) Donorliposomen auf neutrale POPC (1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine) Akzeptorliposomen übertragen, wobei letztere in 5-fachem Überschuss vorliegen. Die Liposomen wurden in Abwesenheit des Proteins inkubiert und neigten während des gesamten Versuchsverlaufs nicht zur Fusion. Nach mehrere Zeitabständen wurden Donor- und Akzeptorliposomen mittels einer Ionenaustausch-Mikrosäule getrennt, die mit CM-Sepharose FF (oder DEAE-Sepharose) gefüllt war. Der Transfer der Arzneistoffe wurde mittels HPLC oder LSC gemessen. Für den Transfer von Paclitaxel wurde eine Zeitkonstante von 0.35 Minuten ermittelt. Diese schnelle Transferrate spricht eher für das „Zusammenstoß-Komplex-Modell“ als für das „Wässrige Diffusions-Modell“. Die Übertragung des Cholesterins zeigte jedoch eine wesentliche langsamere Übertragungsrate. Die ermittelte Zeitkonstante beträgt für negativ-geladene Cholesterin-haltige Liposomen 58 Minuten und für positiv-geladene Liposomen 114 Minuten. Der Zusatz von DOTAP zu den positiv-geladenen Liposomen änderte das Übertragungsverhalten von Cholesterin in einen Zweiphasen-Prozess. Diese Tatsache lässt vermuten, dass der Transfer von Cholesterin auf dem „Diffusions-Modell“

beruht. Die Zeitkonstante, die für die Übertragung von COE ermittelt wurde, beträgt 353 Tage für positiv-geladene und 485 Tage für negativ-geladene Liposomen. Die molekulare Struktur von COE ähnelt der eines Phospholipids. Deswegen hat das COE-Molekül eine hohe Affinität zu Lipidmembranen.

Das zweite Modell basiert auf den Fluoreszenzeigenschaften des Temoporfins. Sogar bei sehr niedrigen Konzentrationen zeigt Temoporfin eine Self-Quenching Effekt. Die Übertragung von Temoporfin von Donor- zu Akzeptorliposomen kann durch die Zunahme der Fluoreszenzintensität zu den verschiedenen Zeitpunkten des in-vitro Transferversuches problemlos gemessen werden. Der Temoporfin-Transfer wurde an 8 liposomalen Formulierungen erforscht, 4 DPPC/DPPE-Formulierungen und 4 DSPC/DSPE-Formulierungen. Es konnte nachgewiesen werden, dass der Temoporfin-Transfer bei einer höheren Temperatur aufgrund der beschleunigten Molekularbewegung und der höheren Lipidfluidität erleichtert ist. Sowohl bei niedrigeren als auch bei höheren Temperaturen zeigten Cholesterin-haltige Formulierungen eine verbesserte Übertragung. Weiterhin wurde beobachtet, dass pegylierte Formulierungen bei niedrigen Temperaturen eine höhere Übertragungsrate aufweisen als die konventionellen Formulierungen.

Contents

Abbreviations

Chol	cholesterol
DCP	dicetyl phosphate
DMPE-PEG 2000	1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)-2000] (ammonium salt)
DOPC	1,2-dioleoyl-sn-glycero-3-phosphocholine
DOPE-PEG	1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy (poly (ethylene glycol) -2000)]
DOPE-Rho	1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl)
DOTAP	1,2-dioleoyl-3-N, N-trimethylammonium-propane (chloride salt)
DPPC	1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine
DPPE-mPEG 2000	1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)-2000]
DPPG	1,2-dipalmitoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (sodium salt)
DSPC	1,2-distearoyl-*sn*-glycero-3-phosphocholine
DSPE-mPEG 2000	1,2-distearyl-phosphatidylethanolamine-methyl-polyethyleneglycol-2000
DSPG	1,2-distearoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (sodium salt)
FRV	freeze-drying rehydration vesicles
HPLC	high performance liquid chromatography
LUV	large unilamellar vesicle
MLV	multilamellar lipid vesicle
PCS	photon correlation spectroscopy
PEG	polyethylene glycol
POPC	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine
PXL	paclitaxel
Rho	rhodamine
SUV	small unilamellar vesicle
TP	temoporfin
[^{3}H] Chol	[1α,2α(n)-^{3}H] cholesterol
[^{3}H] COE	[1α,2α(n)-^{3}H] cholesteryl-oleoyl-ether
[^{14}C] CO	cholesteryl [1-^{14}C] oleate

Part I

INTRODUCTION AND OBJECTIVES

Chapter 1

Introduction

1.1 Liposome-based drug containing formulations

1.1.1 The definition of liposome

Liposomes were discovered in the mid 1960s [Bangham A.D., et al. (1965)] and originally studied as cell membrane models (see Figure 1.1a). Liposomes are formed by the self-assembly of phospholipid molecules in an aqueous environment. Shown schematically in Figure 1.1b, the amphiphilic phospholipid molecules form a closed bilayer sphere in an attempt to shield their hydrophobic groups from the aqueous environment while still maintaining contact with the aqueous phase via the hydrophilic head groups. Liposomes are spherical nano-particulate or colloidal carriers, enclosing an aqueous interior, usually 0.05-5.0 μm in diameter which form spontaneously when certain lipids are hydrated in aqueous media [Bangham A.D. and Horne R.W., 1964]. Small unilamellar vesicles (SUVs) are defined as the liposomes at the lowest limit of size possible for phospholipid vesicles, with a diameter of 0.025 ~ 0.05 μm; large unilamellar vesicles (LUVs) have diameter of the order of 0.1 ~ 0.2 μm; multilamellar lipid vesicles (MLVs) usually consist of a population of vesicles covering a wide range of sizes (0.1-1 μm), each vesicle generally consisting of three or more concentric lamellae. Vesicles composed of just a few concentric lamellae are sometimes called oligo-lamellar liposomes, or paucilamellar vesicles [Menger, F.M., Chlebowski, M.E., etc. (2005)].

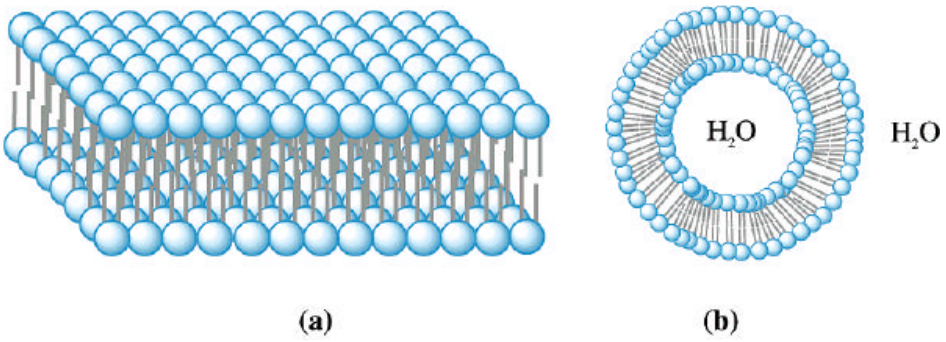

Figure 1.1: *a) Schematic of phospholipids assembled into a bilayer; b) a vesicle, shown in cross section, composed of a bilayer surrounding an aqueous domain [from Menger, F.M., Chlebowski, M.E., etc. (2005)].*

The resulting closed sphere may encapsulate aqueous soluble drugs within the central aqueous compartment (Figure 1.2, left) or lipid soluble drugs within the bilayer membrane (Figure 1.2, centre). Alternatively, lipid soluble drugs may be complexed with cyclodextrins and subsequently encapsulated within the liposome aqueous compartment (Figure 1.2, right) [McCormack B, Gregoriadis G. (1994)]. The encapsulation within/association of drugs with liposomes may alter drug pharmacokinetics, and this can be exploited to achieve targeted therapies. Alteration of the liposome surface is necessary in order to optimize liposomal drug targeting.

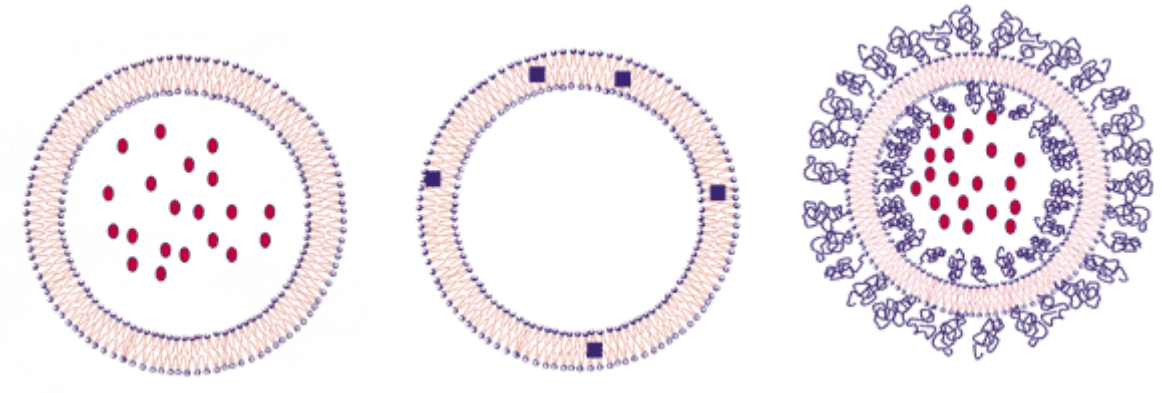

Figure 1.2: *Scheme of different types of drugs incorporated into liposomes: left - aqueous soluble drug encapsulated in aqueous compartment; centre - a hydrophobic drug in the liposome bilayer; right - hydrophilic polyethylenglycol lipids incorporated into liposomes. [from Ijeoma F. Uchegbu, (1999)]*

1.1.2 Liposome preparation methods

Liposomes of different sizes and characteristics can be prepared by very different methods based on several mechanisms operating in the liposome formation. The most simple and widely used method is the thin-film hydration method, by which a heterogeneous population of MLVs is produced. Following further sonication or extrusion through polycarbonate filter membranes, small and more uniformly sized population of SUVs or LUVs can be formed. This method was first characterized by Bangham A.D. and co-workers in 1964, after being used for a long time without being characterized carefully [Bangham A.D. and Horne R.W., 1964]. One of the major disadvantages of this method is its relatively poor encapsulation efficiency (5-15 %) of hydrophilic drugs. The low encapsulation efficiency can be improved to up to 40 % by freeze-drying method. This method involves a dehydration-rehydration procedure of either SUVs or MLVs. Drying brings the lipid bilayers and material to be encapsulated into close contact. Upon re-swelling the chances for entrapment of adhered molecules are larger [Kirby C.J. and Gregoriadis G., (1984)]. This procedure is well suited to prepare liposomal peptide antigens because of its high entrapment efficiency. The encapsulation efficiency can also be increased by the reverse-phase evaporation method. This method was introduced by Papahadjopoulos and co-workers [Papahadjopoulos D. and Watkins J.C., (1967)]. The lipids are hydrated in the presence of an organic solvent. The main drawback of this method is the exposure of the material to be encapsulated to an organic solvent, which might lead to denaturation of proteins. Some other methods like detergent depletion, Ca^{2+} induced fusion or pH adjustment methods are also applied widely in the liposome preparation techniques.

1.1.3 Drug containing liposomal formulations

The parenteral and oral administration of lipophilic drugs is often problematic because of their low water solubility. In order to administer a therapeutic dose of these drugs, formulations containing solubilisers and/or formulations with a high dissolution rate are necessary to deliver the drug. When diacyl-phospholipids with a cylindrical shape are dispersed in water, lipid vesicles comprising a phospholipid bilayer, which surround an aqueous compartment, are formed spontaneously. Therefore, they can encapsulate hydrophilic and bind amphipathic as well as lipophilic drugs [Fahr A., et al. (2005)]. Liposomes are composed of relatively biocompatible and biodegradable materials, and they consist of an aqueous volume entrapped by one or more bilayers of natural and/or synthetic lipids. As one of the most promising nanoparticle technologies, liposomal formulation is particularly

attractive for drug delivery to solid tumours, sites of inflammation and skin permeation. For example, the intercellular tight junctions in the capillaries of most tissues, including the muscle, heart, lung, and connective tissue, produce an effective pore cut-off size of ~6 nm, the majority of tumours exhibit a vascular pore cut-off size of 380-780 nm, sufficient for the enhanced uptake of liposomes [Yuan F., et al., (1995)]. Encapsulation of drugs in a liposome can therefore dramatically alter drug distribution by reducing drug access to normal tissue while facilitating access to tumour tissue through this enhanced permeability and retention effect observed in tumour tissue [Xiang T.X., Anderson B.D., (2006)].

The value of liposomes as model membrane systems derives from the fact that the liposome membrane forms a bilayer structure which is in principal identical to the lipid portion of natural cell membranes. The similarity between liposome and natural membranes can be increased by extensive chemical modification of the liposome membrane, and may be exploited in areas such as drug targeting or immune modulation. The ability of the liposomes both in vivo and in vitro to mimic the behavior of natural membranes, and also to be degraded by the same pathways, makes them a very safe and efficacious vehicle for medical applications [New R.R.C, (1990)]. Liposomes have emerged as important potential drug delivery vehicles for chemotherapy drugs, bioactive lipids and small molecule compounds. The therapeutic activity of these agents can be dramatically improved when their circulation longevity and disease site accumulation properties are increased through liposome encapsulation [Gabizon A. and Papahadjopoulos D., (1988)]. This also offers the advantage of reducing toxicities and other deleterious side effects that are often observed at concentrations similar to, or lower than, those required for maximum therapeutic activity.

Since the introduction of liposomes into the world of intravenous drug delivery research, liposomal formulations for lipophilic drugs have been developed, and successfully introduced in the market [Olson F., et al., (1982); Stamp D. and Juliano R.L., (1979); Gabizon A., et al. (1982)]. Specific examples of such drugs are doxorubicin, daunorubicin, benzoporphyrin (Visudyne®, Verteporfin for injection) and amphotericin B (Albelcet®, AmBisome®) [Prestidge C., et al. (2005)]. In order to rationally design liposomal drug delivery systems, it is necessary to fully characterize their drug retention and release properties both *in vitro* and *in vivo.*

1.2 Drug transfer: its importance and mechanisms

As a unit, the lipid molecule can rotate, rock or diffuse laterally, flip-flop transbilayerly, and engage in interbilayer migration [Lasic, D.D. (1993)]. More importantly,

lateral mobility within one of the two bilayer "leaflets" allows a lipid molecule to drift to (a) a site of action (e.g. to a lipid-dependent protein); (b) accommodate a morphological change (e.g., pseudopod formation or membrane fusion); (c) assemble, along with other membrane components, into domains or "rafts" (see Figure 1.3) [Binder, W.H., Barragan, V., etc., (2003)]. Many factors, including cell type, affect lateral diffusion rates, but they are typically quite fast. Thus, lipids can cross an entire cell surface in a few minutes. Phospholipids can also flip-flop whereby molecules jump from one bilayer leaflet to the other [DeKruijff, B., Zoelen, E.J.J. (1978)]. It may take days for a molecule to flip-flop unless an enzyme catalyzes the process or large amounts of cholesterol are present. Similarly, the exchange rate of lipid molecules between two membrane bilayers is slow: spontaneous interbilayer transfer has half-times ranging from 2 to 24 h or more depending upon the particular lipid and temperature [Roseman, M.A.; Thompson, T.E. (1980); Martin, F.J.; MacDonald, R.C. (1976)].

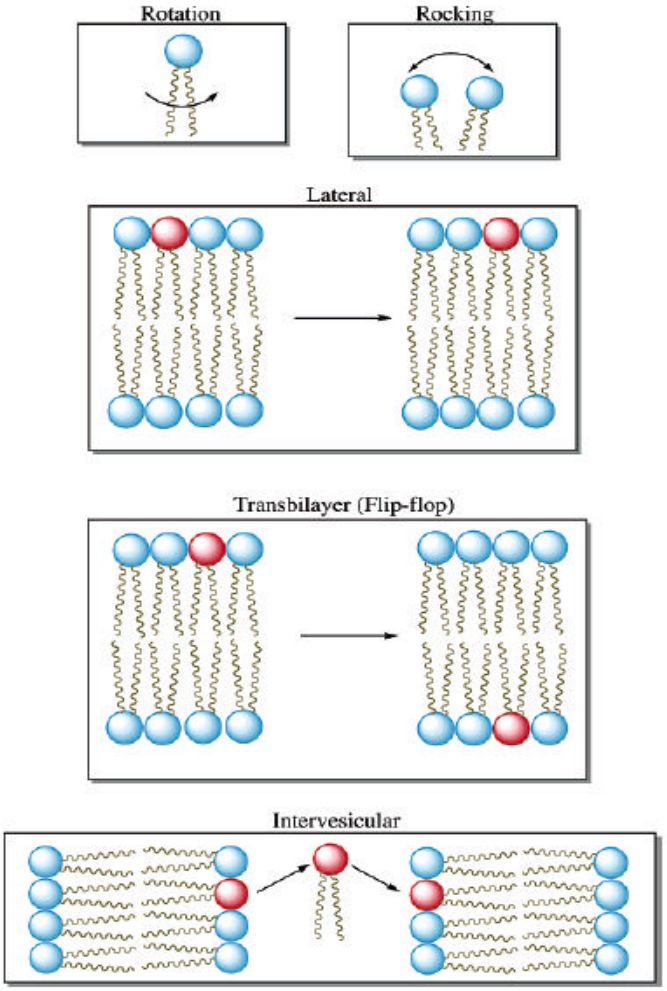

Figure 1.3: *Schematic of various motions possible in a bilayer [from Menger, F.M., Chlebowski, M.E., etc. (2005)].*

An understanding of the mechanisms involved in the transport of drug from tissues to plasma and its subsequent transfer to the liver for degradation is of great importance. It is necessary to develop an *in vitro* model to mimic the *in vivo* transfer of drug in order to explain the transport mechanisms involved. Before studying the drug transfer mechanism, it is fundamental to investigate firstly the lipid exchange or transfer between vesicles. However, numerous complications arise in the investigation of lipid exchange in biological systems due to protein and lipid transfer, adsorption of vesicles to membranes, and the complex structures of biological membranes and lipoproteins [Jonas A. and Maine G.T. (1979); Giraud F. and Claret M. (1979)].

In an appropriate model system, these complications may be minimized so that in the absence of fusion one of two limiting mechanisms may operate in lipid exchange: (i) lipid molecules diffuse through a complex formed by the transient fusion of two lipid monolayers or bilayers following collision of the two particles – collision complex [Gurd, 1960] or (ii) free lipid molecules diffuse through the aqueous phase separating the donor and acceptor particles – aqueous diffusion [Hagerman J.S. and Gould, R.G. (1951)].There is no general agreement on which of these two mechanisms is operative for cholesterol and phosphatidylcholine exchange in biological systems or model membranes [McLean L.R. and Phillips M.C., (1981)]. Smith and co-workers [Charlton S.C., et.al., (1976, 1978); Kao Y.J., et al., (1977); Doody M.C., et al., (1980)] and Roseman M.A. and Thompson T.E. (1980) have established that aqueous diffusion operated for the exchange of fluorescent lipid molecules. On the other hand, phospholipid exchange experiments involving phosphatidylcholine bilayer vesicles have been interpreted in terms of either an aqueous diffusion mechanism [Martin F.J. and MacDonard R.C., (1976); Duckwitz-Peterlein G., et al., (1977); Thilo, (1977); Duckwitz-Peterlein G. and Moraal H., (1978)] or a mechanism involving collisions between the lipid vesicles [Kremer J.M.H., et al., (1977)]

1.3 The existing *in vitro* drug transfer models

There have been all kinds of *in vitro* models to mimic the drug behavior *in vivo.* And it is not uncommon for formulations to exhibit excellent drug retention properties *in vitro,* but to display almost complete drug release within minutes following systemic administration to animals. This calls into question of the usefulness of current *in vitro* pre-clinical drug release assays, and highlights the need for more accurate predictors of true *in vivo* performance. Some of the most important *in vitro* models are summarized in the following paragraphs.

(i) Dialysis based assay

This is one of the most commonly used methods which relies on dialyzing the liposomal formulation against large volumes of buffer at physiological temperatures. The excess buffer is intended to serve as a driving force to promote drug leakage from the liposome. Upon release from the liposomal carrier, free drug crosses the dialysis membrane and accumulates in the buffer system. Serum is frequently added to the dialysis buffer to more closely mimic the physiological environment (proteins and lipid constituents) that liposomes encounter *in vivo* [Allen T.M. and Cleland L.G. (1980)].

It has been reported that serum albumin enhances diffusion of [^{14}C] glucose from phosphatidylcholine-cholesterol-dicetylphosphate multilamellar vesicles at pH 3.5, but not at pH 7.0 [Sweet C. and Zull J.E., (1970)]. An enhanced leakage of sucrose from phosphatidylcholine liposomes induced by heparinized blood or by serum albumin has also been reported [Zborowski J., et al., (1977)]. And also, during incubation with 50-66% plasma, phosphatidylcholine from liposomes was transferred to high density lipoproteins in a one-way process with resulting release of entrapped ^{125}I-labeled albumin from the liposomes [Scherphof, G., et al., (1978)]. There has been the report that the presence of 10% fetal calf serum dramatically increased release in the region of the phase transition from diapalmitoylphosphatidylcholine-distearoylphosphatidylcholine (3:1) vesicles. The presence of 10% fetal calf serum had little effect at temperatures below the phase transition temperature [Yatvin M.B., et al., (1978)]. However, despite of these efforts, the drug leakage properties observed using dialysis-based systems often show poor correlation between *in vitro* and *in vivo* results.

(ii) MLV based assay

In this assay, drug-encapsulated large unilamellar vesicles (LUVs) in diameter of ca. 100 nm are incubated with a 100-fold excess of multilamellar vesicles (MLVs) containing 300 mM sucrose, which served as “acceptors” for drug release and transfer from “donor” LUVs, and are intended to mimic the vast membrane pool present in physiological membranes. Following incubation at 37°C, the donor and acceptor populations are separated with greater than 90% efficiency by centrifugation at 1600 x g for 10 min. The amount of drug in the MLV pellet reflects the degree of drug leakage from the donor to acceptor liposomes. A schematic illustration of this method is demonstrated in Figure 1.4.

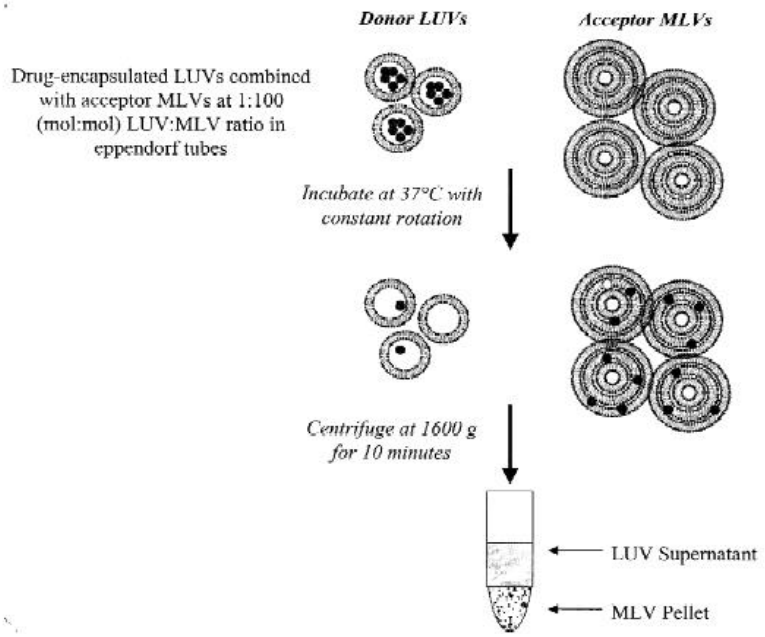

Figure 1.4: *Schematic illustration of the in vitro drug release assay procedure [Shabbits J. A., et al., (2002)].*

This assay was first developed by Shabbits J.A. and co-workers [Shabbits J. A., et al., (2002)] and the transfer of doxorubicin and verapamil was investigated. Drugs, lipids were labeled with [^{3}H] or [^{14}C], liposome concentrations were determined by scintillation counting. The release of encapsulated doxorubicin and verapamil from liposomes was compared with the dialysis assay and the *in vivo* animal based assay. The MLV-based drug release assay showed a better prediction of *in vivo* drug transfer. Often, in vitro drug release assays do not accurately predict the liposomal drug retention properties observed in vivo. One postulation is that this discrepancy is due to the large membrane pool present in blood cells and tissues, into which drugs can be distributed after in vivo administration. Therefore, this in vitro drug release assay could more accurately predict in vivo drug release from liposomes following systemic administration.

(iii) Cell based assay

In this model, the substances of interest are incorporated into cells during growth. The cells are incubated in acceptor vesicles in excess. After the separation, the transfer of the substances is measured. In such a cell based assay published by Rottem and co-workers [Rottem S., et al., (1981)], labeled cholesterol was incorporated into *Acholeplasma laidlawii* and *Mycoplasma gallisepticum* cells during the cell growth. The cells were incubated with a large excess of phosphatidylcholine vesicles containing unlabeled cholesterol at a 1:1 molar ratio. The transfer of cholesterol during the incubation time was only detected after the

addition of bovine serum albumin (BSA). BSA could enhance considerably cholesterol exchange, possibly by increasing cholesterol solubility in the aqueous medium. An interpretation of two cholesterol pools was raised according to the exhibited biphasic transfer curve. A rapidly exchangeable pool representing cholesterol located in the outer leaflet of the bilayer, and a slower exchangeable pool that represents cholesterol in the inner leaflet of the bilayer [Rottem S., et al., (1981)]. Another hypothesis is that the biphasic cholesterol exchange kinetics does not result from the transbilayer distribution of cholesterol, but reflects the presence in the membrane of two cholesterol pools associated with lipids of high and low affinity for cholesterol. In the case of *Acholeplasma laidlawii*, the lipids of low affinity for cholesterol are glycolipids, while phospholipids constitute lipids of high affinity for cholesterol [Davis P.J., et al., (1984)].

This assay is more complicated compared with the other *in vitro* assays. The type of drugs studied is limited. Up to now, only cholesterol is reported to be incorporated into the cells, due to the requirement of cholesterol for the growth of the *Mycoplasma gallisepticum* cells. And also the utilization of the cells brings difficulty to this assay.

(iv) Agarose gel based assay

The system is prepared in a 1.2 cm by 5.0 cm vial by first depositing the bottom layer containing the liposomes in a 1% w/v agarose solution (at 48°C) and allowing this to gel. At room temperature the agarose solution is hardened into a semi-solid matrix within 20 min. The top layer which consists of a 2% w/v agarose solution is then placed on the 1% agarose layer and is allowed to gel. The two layers are visible in Figure 1.5. The receptor compartment contains 4.5 ml of HBS. Serum proteins could also be added to the receptor phase to mimic the body environment. The receptor solution was completely replaced at various time points and the amount of marker released from the matrix and liposomes was determined. Under these conditions the agarose matrix remained intact throughout the duration of the experiment. A photography of the agarose matrix *in vitro* release system in the bottom of glass vials is illustrated in Figure 1.5.

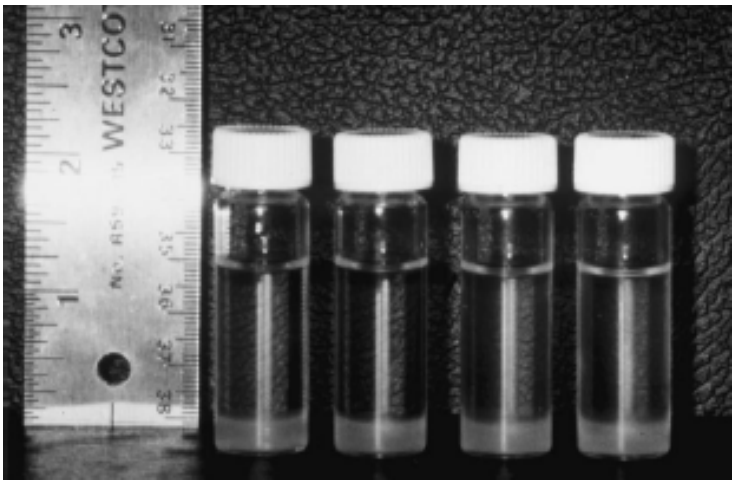

Figure 1.5: *Photograph of the agarose matrix in vitro release system in the bottom of glass vials [from Peschka R., Dennehy C., etc. (1998)].*

This assay was presented by Peschka R. and co-workers to study the release kinetics of liposome encapsulated material in the presence of biologic components. This *in vitro* model is a convenient and reproducible system that permits the study of the release of high molecular weight molecules such as proteins from liposomal formulations in the presence of serum. Furthermore, the model is not restricted to liposomal preparations but may be applied to other colloidal drug delivery systems such as microspheres and emulsions.

(v) Biotin-streptavidin interaction based assay

Streptavidin is a tetrameric protein purified from Streptomyces avidinii that binds very tightly to the vitamin biotin with a K_d of 10^{-14} mol/L. Biotin-streptavidin interaction is one of the strongest biological and non-covalent interactions known. This interaction is widely taken advantage in many scientific laboratories.

In this assay acceptor liposomes are prepared as biotinylated liposomes. Donor liposomes contain the lipids or drugs of interest. The streptavidin is immobilized on superparamagnetic iron oxide particles (SA magnetic particles). Due to the well characterized biotin-streptavidin interactions, the biotinylated acceptor liposomes can be bound on to the SA magnetic particles. Unbound donor liposomes in the supernatant can be removed from the mixture after separation using a magnetic separator. SA magnetic particles with bound acceptor liposomes are then collected after several washes and the percentage transfer of lipids or drugs from donor to acceptor is measured by suitable techniques.

This assay was first developed by Li W.M. and co-workers [Li W.M., et al., (2001)] for studying the transfer of polyethylene glycol (PEG)-modified phosphatidylethanolamine. As

measured by this assay, it is found that an increase in acyl chain length from C14:0 to C16:0 of the PEG-lipid resulted in a significant reduction in the rate of transfer. The results are found to be comparable under *in vivo* and *in vitro* conditions. Furthermore, by using this biotin-streptavidin interaction, monoclonal antibodies are coupled to biotin residues of the liposomes resulting liposome-antibody complexes.

(vi) Ion-exchange column based assay

The ion-exchange mini-column model is an in vitro system for measuring the transfer of lipophilic drug molecules from the liposomal carrier system to model membranes mimicking other membranous binding places in the body (erythrocyte membranes, endothelial cell membranes, LDL, etc.). It consists of the insertion of the drug into negatively (or positively) charged liposomes using standard techniques. Neutral liposomes (mostly PC-liposomes) are used as acceptor medium at an excess in relation to the donor liposomes. After mixing the two liposome populations together, samples are processed at defined time points over an ion-exchange column, which allows only the neutral (acceptor) liposomes to be eluted. This model was first presented by Hellings and co-workers [Hellings J.A., et al., (1974)] and was afterwards modified by van den Besselar and co-workers [van den Besselaar A.M.H.P., et al., (1975)]. The analysis of the amount of drug in the acceptor liposomes is either done by HPLC, Fluorescence or most easily by radioactive tracers. A schematic graph of the *in vitro* ion-exchange micro-column model is illustrated in Figure 1.6.

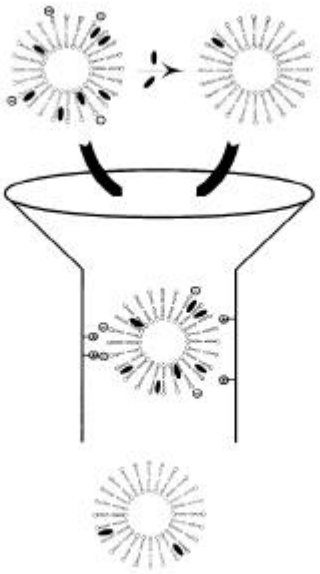

Figure 1.6: *The schematic illustration of the liposomes separation procedure by using in vitro ion-exchange micro-column model [from Fahr A. and Seelig J., 2001].*

By using this model, the mechanism of transfer of cholesterol and phosphatidylcholine was investigated by McLean L.R. and Phillips M.C. in 1981. It was found that the transfer or exchange half-time of cholesterol and POPC phospholipid is 2.3 ± 0.3 h and 48 ± 5 h respectively. The results obtained are consistent with a mechanism of lipid exchange in which cholesterol and phosphatidylcholine diffuse through the aqueous phase; the experimental activation energy is associated with desorption of lipid from the donor bilayer into the aqueous phase.

This ion-exchange column based assay is explicitly simple and easy to handle. Compared to all the other assays introduced above, the most important advantage of this assay is that it is possible to study the transfer kinetics of drugs during the initial incubation time. For all the other models, a necessary period of incubation time is required. These methods therefore do not allow the investigation of the extremely fast transfer kinetics of some lipophilic drugs. For example, a transfer half-time of only 4.5 min was reported in the transfer of cyclosporin A between the liposomes [Fahr A. and Seelig J., 2001]. In this case, the ion-exchange column based assay is the only possibility among all the mentioned assays for studying the transfer kinetics.

Due to the attractive advantages of the ion-exchange column assay, this model was finally selected as the *in vitro* model for the transfer study of paclitaxel, cholesterol and cholestoryl-oleoyl-ethyl between the liposomal membranes in the presented study.

1.4 The drugs used in the experiments

The drugs investigated for the transfer experiment are introduced as follows. Paclitaxel (PXL, see Figure 1.7 for the molecular structure) is one of the best antineoplastic drugs found from nature in the past decades, which has significant effects against a wide spectrum of cancers, especially ovarian cancer, breast cancer, colon cancer, small and non small lung cancer, and AIDS-related Kaposi's sarcoma [Adams J.D., et al., (1993)]. Unfortunately, clinical development of this drug has been hampered by its poor aqueous solubility, which necessitates administration of the drug in Diluent 12 (Cremophor EL and ethanol; 1:1 v/v) [Internet: http://www.taxol.com/index.html]. This formulation causes serious side effects, such as hypersensitivity reactions, nephrotoxicity, neurotoxicity, and cardiotoxicity [Zhao L.Y. and Feng S.S., (2005)]. Encapsulation of PXL by liposomes provides an environment that may enhance the solubility of PXL, achieve a controlled and targeted delivery of the drug, and, more importantly, avoid the use of the toxic adjuvant and reduce chronic toxicity of the drug [Zhao L.G, et al., (2004)]. To maximize its pharmacological potential, the stability of PXL-loaded liposomes is vital. It is thus very important to have an in-depth understanding of

the state of PXL in the lipid bilayer and the nature of the molecular interactions between PXL and phospholipids. Such an investigation will lend insight not only to the therapeutic performance of PXL-liposome formulations but also to the pharmacokinetic effects of PXL. Cationic liposomes are widely used for DNA/gene therapy and they have a positive zeta-potential that results in their nonspecific interaction with a wide range of anionic proteins and non-target cells [Ding AH, Porteu F (1990)]. DOTAP, as one of the most widely used cationic lipids, enhances their interaction with cellular membranes [Farhood H., et al., (1995)]. Besides, the interaction of cationic lipids with the membranes may change the structure and the properties of membranes [Zhao and Feng, (2004)]. As a result, the commercially available PXL containing liposomes are positively charged.

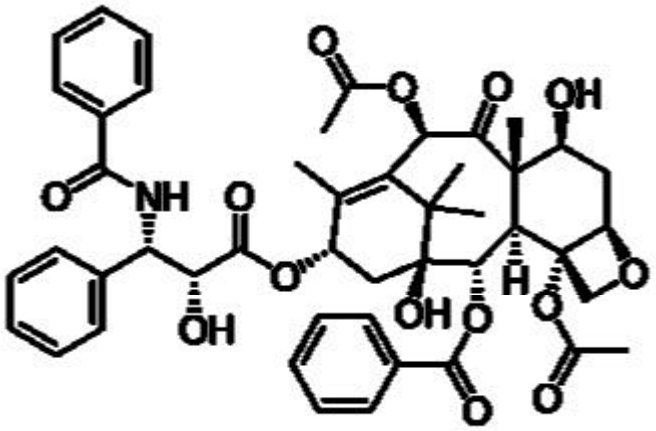

Figure 1.7: *Molecular structure of paclitaxel.*

Since the interaction between sterols and phospholipids is the most extensively investigated lipid-lipid interaction in membranes [Bloch K.E., (1983)], cholesterol (see Figure 1.8 for the molecular structure), as a sterol alcohol that is essentially insoluble in aqueous solutions, was chosen as a comparison to the transfer of PXL. is essentially insoluble in aqueous solutions. In mammals, Cholesterol is normally soluble by its association with other lipids, such as phospholipids or bile acids; thus, most cholesterol is found in cell membranes, plasma lipoproteins, and bile [Mouristen O.G. and Zuckermann J.J. (2004)]. Most of cholesterol is synthesized by the liver and other tissues, including the adrenal glands and reproductive organs. Some cholesterol is absorbed from dietary sources. The most common sterol of eukaryotes is a key constituent of cell membranes and works as the precursor of bile acids, cholecalciferol (vitamin D) and steroid hormones including cortisol, cortisone, aldosterone and sex hormone progesterone in vertebrates [Smith L.L., (1981)].

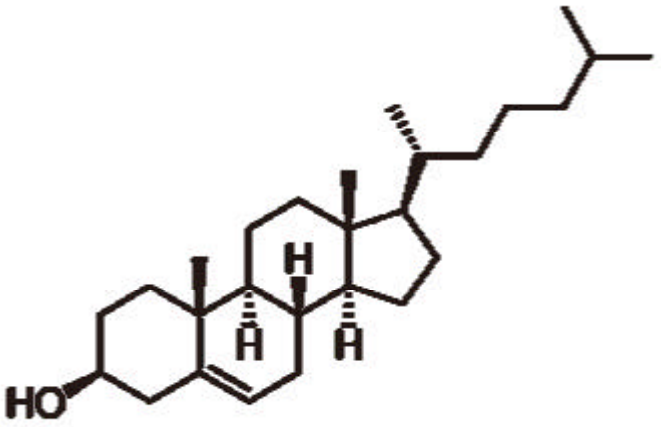

Figure 1.8: *Molecular structure of cholesterol.*

A cholesterol derivative, cholesteryl-oleoyl-ether (COE), is selected as a comparison drug to the transfer of PXL and Cholesterol. It is known that the transfer of this cholesterol derivative is extremely slow, and therefore it can be used as a non-exchangeable label during the lipid transfer [Fahr A. and Seelig J., (2001)].

Foscan® or meta-tetra (hydroxyphenyl) chlorin (Temoporfin, see Figure 1.9 for the molecular structure) is a member of the chlorin family and belongs to the second-generation photosensitizer [Bonnett R. et al., (1989)]. The drug is one of the most effective sensitizers studied to date [Dougherty T.J. et al., (1998)]. It mediates cell photo-damage principally through singlet oxygen formation [Melnikova V.O., et al., (1999)] and its tumoricidal effect appears to be very sensitive to oxygenation conditions [Coutier S., et al., (2002); Coutier S., et al., (2001)]. Foscan® has been granted of European approval for palliative treatment of patients with advanced head and neck cancers. Recent clinical open-label multi-center studies also reported the efficacy of Foscan®-PDT in the treatment of early squamous cell carcinoma [Copper M.P., et al., (2003); Hopper C., et al., (2004)].

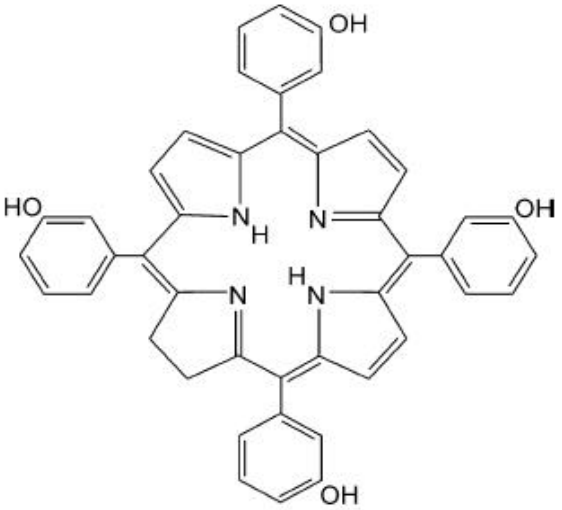

Figure 1.9: *Molecular structure of Temoporfin.*

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Chapter 2

Objectives

The parenteral and oral administration of lipophilic drugs is often problematic because of their low water solubility. Liposomes are composed of relatively biocompatible and biodegradable compounds, and they consist of an aqueous volume entrapped by one or more bilayers of natural and/or synthetic lipids. Therefore, liposomal formulations are considered as one of the most promising nanoparticle technologies for drug delivery to solid tumors, sites of inflammation and skin permeation. An understanding of the mechanisms involved in the transport of drug from tissues to plasma and its subsequent transfer to the liver for degradation is of great importance. It is necessary to develop an *in vitro* model to mimic the *in vivo* transfer of drug in order to explain the transport mechanisms involved. The present study deals with the investigation of the factors which influence the transfer of lipophilic drugs and the relationship with the mechanisms in the drug transfer. The present work has been structured in different parts in order to achieve the following particular objectives.

2.1 Ion-exchange micro-columns based *in vitro* model

The first model is based on the ion-exchange micro-columns. The transfer kinetics of three compounds, paclitaxel, cholesterol and cholesteryl-oleoyl-ether (COE), has been investigated. Before the drug transfer experiment, the capacity and reliability of the *in vitro* ion-exchange micro-column model has to be determined and the optimized parameters are selected for the drug transfer experiment. Under the selected conditions, the drugs are transferred from positively (or negatively) charged, unilamellar DOPC (1,2-Dioleoyl-sn-Glycero-3-phosphocholine C18:1/C18:1) donor vesicles to 5 times excess of neutral POPC

(1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine C16:0/C18:1) acceptor vesicles. Vesicles were incubated in the absence of protein and were stable to fusion over the course of the experiment. At intervals, donor and acceptor vesicles were separated by passing through a column filled with CM Sepharose FF (or DEAE-Sepharose); slight amount of the charged and 80-95 % of the neutral vesicles were recovered in the eluate. This part of the research project has been conducted in collaboration with MediGene Company (Munich, Germany), where the HPLC quantification of paclitaxel transfer has been performed. The transfer kinetics results of paclitaxel, cholesterol and COE will be compared and discussed. The possible factors and mechanisms which influence, or decide, the compound transfer will be investigated. These results will provide comprehensive insights into the molecular interactions of the compounds, i.e. paclitaxel, cholesterol and COE, with the phospholipids in the domains of bilayer vesicles and their possible effects on the loaded liposomal formulations.

2.2 Fluorescence based *in vitro* model

The second model is based on the fluorescence character of the drug temoporfin. The transfer of temoporfin from donor to acceptor liposomes can be easily measured by the fluorescence intensity at different time points in the *in vitro* drug transfer experiment since the fluorescent substance shows a self-quenching effect of the fluorescence at a high concentration. The transfer of temoporfin from eight temoporfin-containing C16 and C18 liposomal formulations will be investigated. These results are used to explain the possible factors that influence the transfer kinetics of the drug between liposomal membranes and the mechanisms behind. Moreover, the effect of cholesterol and PEG to the lipid bilayers will also be explored.

Part II

EXPERIMENTAL

Chapter 3

Preparation and characterization of liposomes

3.1 Materials

DOPC (1,2-Dioleoyl-sn-Glycero-3-phosphocholine C18:1/C18:1), DOPE-Rho (1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl)), DOPE-PEG (1,2-Dioleoyl-*sn*-Glycero-3-Phosphoethanolamine-N-[Methoxy (Poly (ethylene glycol) -2000)] C18:1/C18:1), DPPC (1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphocholine C16:0/C16:0), DPPG (1,2-Dipalmitoyl-*sn*-Glycero-3-[Phospho-*rac*-(1-glycerol)] (Sodium Salt) C16:0/C16:0), DSPC (1,2-Distearoyl-*sn*-Glycero-3-Phosphocholine C18:0/C18:0), DSPG (1,2-Distearoyl-*sn*-Glycero-3-[Phospho-*rac*-(1-glycerol)] (Sodium Salt) C18:0/C18:0) and DMPE-PEG 2000 (1,2-Dimyristoyl-*sn*-Glycero-3-Phosphoethanolamine-N-[Methoxy(Polyethylene glycol)-2000] (Ammonium Salt)) were purchased from Avanti Polar Lipids (Alabaster, AL, USA); DOTAP (1,2-dioleoyl-3-N, N-trimethylammonium-propane (chloride salt)) was obtained from Merck Eprova (Schaffhausen, Switzerland); Dicetyl phosphate (DCP) and cholesterol (≥ 99%) was from Sigma Chemical (St. Louis, MO, USA); POPC (1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine C16:0/C18:1), DPPE-mPEG 2000 (1,2-Dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000]) and DSPE-mPEG 2000 (1,2-Distearyl-phosphatidylethanolamine-methyl-polyethyleneglycol-2000) were obtained from Genzyme Phermaceuticals (Liestal, Switzerland); Paclitaxel was from Cedarburg Pharmaceuticals (Grafton, WI, USA) and Temoporfin (meta-tetrahydroxyphenylchlorin) was provided as a kindly gift from Biolitec AG (Jena, Germany).

Tris Ultra Quality (Tris-(hydroxymethyl)-aminomethan, ≥99.9%) was purchased from Carl Roth GmbH (Karlsruhe, Germany). The preparation of tris buffer at different concentrations was obtained by dissolving appropriate amount of Tris Ultra Quality from Carl Roth GmbH (Karlsruhe, Germany) in Milli-Q water and adjusted to a final pH at 7.4 by addition of HCl. A 691 pH meter, from Ω Metrohm (Herisau, Switzerland) was used for the pH measurement. All the buffers were degassed by sonicating for 10 min, and stored in the refrigerator at 4 °C. The water used in this research was purified by a Milli-Q Direct-Q® water purification system from Millipore Corporation (Billerica, MA, USA), and had a purity with a resistance of 18.2 MΩ. Chloroform (≥99.8%, HPLC grade, 1.5L) and triton X-100 (laboratory grade) were supplied by Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Hydrochloric acid fuming 37% (AR grade) was from Merck KgaA (Darmstadt, Germany). Methanol (≥99.9%, HPLC/Spectro grade) was obtained from Tedia Company, Inc (Fairfield, OH, USA).

Two ion-exchange gel materials CM Sepharose ™ Fast Flow and DEAE Sepharose ™ CL-6B preserved in 500 mL 20 % ethanol solution were ordered from GE Healthcare Bio-Sciences AB (Uppsala, Sweden). Radioactive substances [1α,2α(n)-^{3}H] Cholesteryl-oleoyl-ether, Cholesteryl [1-^{14}C] oleate and [1α,2α(n)-^{3}H]Cholesterol (all of them are 99.6%) were purchased as stock solutions in toluene solvent from GE Healthcare UK Ltd (Amersam radiochemicals) (Buckinghamshire, UK). Rotiszint eco plus (for hydrophilic and hydrophobic samples) were purchased from Carl Roth GmbH (Karlsruhe, Germany) for radioactivity counting. All chemicals were of highest available purity and used without further purification.

3.2 Preparation of donor, acceptor and saturation liposomes

The preparation of the donor and acceptor liposomes was carried out by the thin-film hydration method in combination with extrusion through polycarbonate membrane, and the preparation of the saturation liposomes was achieved by the thin-film method in combination with sonication via a sonicator under nitrogen in order to reduce the obtained MLVs to LUVs or SUVs, respectively.

Donor PXL containing liposomes used in the transfer experiment were prepared in the same way as the commercially available PXL containing liposomes manufactured by MediGene Company as cationic liposomes. Liposomes were prepared from DOPC lipids. Addition of DOTAP into lipids gives rise to a positive charge. Paclitaxel, as a lipophilic drug, is supposed to be in the bilayer of the liposomes. The liposomes were labeled with Rho-DOPE as a non-exchangeable label to trace the recovery of donor liposomes. No cholesterol was added into

the lipids in order to avoid the possible influence of cholesterol, which may occupy the non-specific binding places of paclitaxel in the lipids.

It has been found that the amphiphilic polyethylene glycol (PEG) derivative has a long-circulation property to the clearance of the liposomes from bloodstream [Leverman P., et al., (2001)]. Therefore, the transfer kinetics of PXL with and without the incorporation of DOPE-PEG in lipid membranes was investigated with the purpose to study the influence of PEG in the drug transfer.

Donor liposomes for cholesterol (Chol) or cholesteryl-oleoyl-ether (COE) transfer study were prepared as both positively and negatively charged liposomes. Cholesterol and cholesteryl-oleoyl-ether were labeled as [1α,2α(n)-^{3}H]Cholesterol ([^{3}H] Chol) and [1α,2α(n)-^{3}H] Cholesteryl-oleoyl-ether ([^{3}H] COE), respectively, in a final concentration of 1 μCi/ml of liposome suspension. A certain amount of cholesterol was incorporated into the lipids in order to improve the properties of the liposomes.

Donor liposomes for temoporfin (TP) transfer study were prepared from two main types of lipids, i.e. DPPC (1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphocholine C16:0/C16:0) and DSPC(1,2-Distearoyl-*sn*-Glycero-3-Phosphocholine C18:0/C18:0). Additionally, cholesterol and/or DOPE-PEG were incorporated into the lipids.

Acceptor liposomes for all the four drug transfer studies were prepared from POPC (1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine C16:0/C18:1) lipids. Some radioactive acceptor liposomes were prepared by tracing lipids with Cholesteryl [1-^{14}C] oleate ([14C] CO) as a non-exchangeable marker [Bar L.K. et al., (1986)]. Acceptor liposomes were prepared according to the composition of the drug containing donor liposomes except for the temoporfin transfer study, where only pure POPC liposomes were used.

Saturation liposomes were used only for ion-exchange micro-columns based drug transfer experiments, i.e. only paclitaxel, cholesterol and cholesteryl-oleoyl-ether transfer experiments, and were prepared in the same composition as the respective acceptor liposomes. A suitable amount of the saturation liposomes was applied to the columns to reduce non-specific adsorption and improve the recovery of acceptor vesicles [McLean L.R. and Phillips M.C., (1981)].

3.2.1 Composition of liposomes

All the liposomal formulations and their lipids composition are summarized in Tables 3.1 and 3.2 expressed in mol/mol and mg/mg, respectively.

Table 3.1: *Donor, acceptor and saturation liposomal formulations and their lipids composition used in paclitaxel, cholesterol, and cholesteryl-oleoyl-ether transfer experiments (the ion-exchange micro-column based experiment).*

Liposome types	Liposomal formulations ([lipids]=10 mM)	Composition ratios (mol/mol)
Donor liposomes for PXL transfer study	DOTAP/DOPC/DOPE-Rho	25/74/1
	DOTAP/DOPC/DOPE-Rho/PXL	25/71/1/3
	DOTAP/DOPC/DOPE-Rho/DOPE-PEG	25/69/1/5
	DOTAP/DOPC/DOPE-Rho/DOPE-PEG/PXL	25/66/1/5/3
	DOTAP/DOPC/DOPE-Rho	20/79/1
	DOTAP/DOPC/DOPE-Rho	10/89/1
	DOTAP/DOPC/DOPE-Rho	5/94/1
Donor liposomes for Chol transfer study	DOTAP/DOPC/Chol/[^{3}H]Chol[1]	25/72/3
	DCP/DOPC/Chol/[^{3}H]Chol[1]	2/7/1
Donor liposomes for COE transfer study	DOTAP/DOPC/Chol/[^{3}H]COE[1]	25/72/3
	DCP/DOPC/Chol/[^{3}H]COE[1]	2/7/1
Acceptor liposomes for PXL transfer study	POPC[2]	100
	POPC/[^{14}C] CO[1,2]	100
Acceptor liposomes for positively charged Chol and COE transfer study	POPC/Chol/[^{14}C] CO[1]	97/3
Acceptor liposomes for negatively charged Chol and COE transfer study	POPC/Chol/[^{14}C] CO[1]	8/2
Saturation liposomes for PXL transfer study	POPC	100
Saturation liposomes for positively charged Chol and COE transfer study	POPC/Chol	97/3
Saturation liposomes for negatively charged Chol and COE transfer study	POPC/Chol	8/2

1. The final radioactivity was 1μCi/ mL liposome preparation

2. Lipids concentration was 50 mM

Table 3.2: *Donor, acceptor liposomal formulations and their lipids composition used in temoporfin transfer experiment.*

Liposome types	Liposome Abbreviations	Lipids composition([lipids]=20 mg/mL)	Composition ratios (mg/mg)
Donor liposomes for TP transfer study	C16	DPPC / DPPG	18.0 / 2.0
	C16_TP	DPPC / DPPG / TP	18.0 / 2.0 / 1.5
	C16_TP_P	DPPC / DPPG / TP / DSPE-mPEG 2000	18.0 / 2.0 / 1.5 / 4.6
	C16_TP_Chol	DPPC / DPPG / TP / Chol	18.0 / 2.0 / 1.5 / 0.9
	C16_TP_P_Chol	DPPC / DPPG / TP / DSPE-mPEG 2000 / Chol	18.0 / 2.0 / 1.5 / 4.6 / 0.9
	C16_TP_DOTAP	DPPC / DOTAP / TP	18.0 / 2.0 / 1.5
	C18	DSPC /DSPG	18.0 / 2.0
	C18_TP	DSPC / DSPG / TP	18.0 / 2.0 / 1.5
	C18_TP_P	DSPC / DSPG / TP / DSPE-mPEG 2000	18.0 / 2.0 / 1.5 / 4.6
	C18_TP_Chol	DSPC / DSPG / TP / Chol	18.0 / 2.0 / 1.5 / 0.9
	C18_TP_P_Chol	DSPC / DSPG / TP / DSPE-mPEG 2000 / Chol	18.0 / 2.0 / 1.5 / 4.6 / 0.9
Acceptor liposomes for TP transfer study	Accep.	POPC/Chol	20.0 / 0.9

3.2.2 Liposome preparation procedures

All the substances, weighed accurately on a microbalance, were prepared as stock solutions in chloroform (only temoporfin stock solution was prepared in methanol due to its poor solubility in chloroform) and stored at –20 °C. All the stock solutions were in the concentration of 20 mg/mL except for the donor liposomes for PXL transfer study, where the stock solutions were at a concentration of 2 mg/mL. Radioactive substances were purchased as a stock solution in toluene. [1α,2α(n)-^{3}H] Cholesteryl-oleoyl-ether and [1α,2α(n)-^{3}H]Cholesterol stock solutions were in a concentration of 1μCi/μL, and Cholesteryl [1-^{14}C] oleate stock solution was in a concentration of 100 mCi/μL. Appropriate amounts of stock solutions were taken into a 50 ml round-bottomed flask. The stock solution was dried at a temperature above the gel-to-liquid crystalline phase transition of the lipid (T_m) by using a Rotavapor Büchi R-114, (Essen, Germany) with a constant rotating speed of 60 rpm. A gentle vacuum of 200 mbar (except for TP containing liposomes, where a pressure gradient of 500-530 mbar, 5 min; 200 mbar, 10 min; 10-30 mbar, 45-90 min was used) was achieved by a vacuum pump Vacobox Büchi B-177, (Essen, Germany) during 30 minutes. Then, the residuary organic solvents were further removed at 2 mbar for another 60 minutes (no exhalation of chloroform indicates a successful removal of chloroform). The temperatures

were controlled by using a water bath Büchi B-481, (Essen, Germany). The formed lipid thin-film was hydrated by addition of pure Milli-Q water or 5 % glucose to obtain the respective lipids concentration. The reconstitution procedure lasted 60 min under rotation (60 rpm) at a normal pressure. The reason of choosing Milli-Q water for the hydration of lipid thin-film can be explained as follows: the highest chemical stability of PXL is at pH range 3-5 [Dordunoo S. and Burt HM, (1996)]. Considering a decreased stability of PXL at higher pH and to avoid the influence of buffer, Milli-Q water, with the pH at around 6, was selected for the hydration of PXL containing liposomes. In order to be in accordance with PXL containing liposomes, Milli-Q water was decided for all the preparations (i.e. PXL, Chol and COE containing donor liposomes and the acceptor liposomes) in the ion-exchange micro-column model based drug transfer study. The 5 % glucose solution was used for hydration of lipid thin-film in TP containing formulations in order to be in accordance with the commercial products from Biolitec AG. Glucose, as a monosaccharide, has a relative low collapse temperature and is widely used as a cryoprotective substance. It has been chosen by Biolitec AG as the hydration solution during the preparation of TP lyophilised formulations.

The obtained MLVs suspension was then equilibrated at room temperature in the dark for a certain time. This caused the newly formed multilamellar structure to be completely hydrated. For donor liposomes in PXL transfer study, this equilibration procedure was confined to only 15 min due to the degradation potential of PXL. As we know, PXL is almost insoluble in water and is susceptible to hydrolysis. In the case of Chol or COE containing donor liposomes and acceptor liposomes, an equilibration time of 2 h was employed. For the preparation of TP containing liposomes, this duration was increased to 16 h in order to assure a thoroughly equilibration. For the preparation of TP containing liposomes, the organic solvents were evaporated under a pressure gradient (500-530 mbar, 5 min; 200 mbar, 10 min; 10-30 mbar, 45-90 min) and with the temperature at 50 °C for DPPC liposomes, and 65 °C for DSPC liposomes.

The reduction of MLVs to LUVs (Large unilamellar vesicles) was achieved by 21 times of extrusion (only 11 times for PXL containing liposomes) across a pore diameter 200 nm (100 nm for donor and acceptor liposomes for TP transfer study) polycarbonate membrane ARMATIS, 19 mm in diameter, (Schriescheim, Germany) in a Liposofast manual mini-extruder (Avestin, Ottowa, Canada) with two 1000 µl syringes from Hamilton Company, (Bonaduz, Switzerland). A photograph of the extrusion of liposomes by using LiposoFast-Basic manual mini-extruder is illustrated in Figure 3.1.

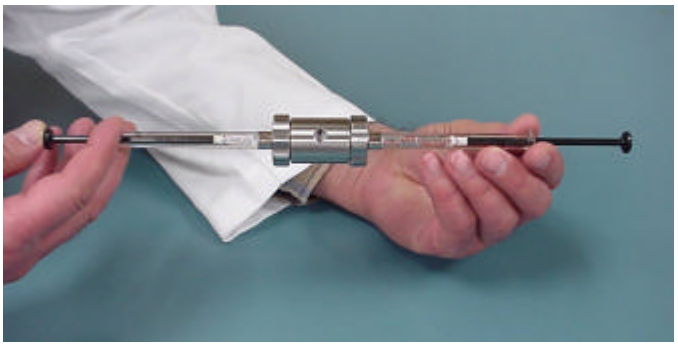

Figure 3.1: *The extrusion of liposome by using LiposoFast-Basic manual mini-extruder. Picture was from Avestin website http://www.avestin.com/lf.html#lfbasic.*

During the whole procedure, a temperature of 4 °C was strictly controlled for the preparation of donor liposomes for PXL transfer study to avoid the degradation of PXL. A temperature of 30 °C was used for the preparation of all the POPC liposomes and donor liposomes for Chol and COE transfer study. A much higher temperature above the phase transition temperature of the lipids was selected for the preparation of donor liposomes for TP transfer study, i.e. 50 °C for DPPC liposomes and 65 °C for DSPC liposomes. The round-bottomed flask for donor liposomes for PXL and TP transfer study was always protected by aluminium foil to avoid the possible bleaching of the fluorescence. One set of instruments was used exclusively for radioactive products. The freshly prepared liposomes were stored under N_2 at 4 °C, protected from light and consumed within one week (PXL containing liposomes has to be prepared and used on the same day).

The saturation liposomes were small unilamellar vesicles (SUVs). The reduction of MLVs to SUVs was achieved by ultrasonic irradiation method. A probe sonicator (Soniprep 150 MSE, Zitec AG, Oberwil, Switzerland) with a process timer (Zitec AG, Oberwil, Switzerland) was used to break down multilamellar liposomes to small unilamellar vesicles. The obtained MLVs suspension was transferred into a special 3 ml conical glass tube, which was placed into a 0 °C water-bath. The tip of the sonifier was immersed into the sample adjusting it accurately to at least 1 cm above the bottom of the vessel. Nitrogen gas was purged during the whole time for avoiding oxidation of the liposomes. The timer was set to 60 minutes with 50 % effective sonication time, each cycle lasting 30 seconds. The metallic particles (titanium) shed from the tip and large particles were removed by centrifugation with a simple desktop centrifuge at 1100 g for 15 min. A liposome suspension with radii around 30-60 nm was obtained with a small percentage of MLVs. The presence of MLVs was helpful for the saturation of the column materials, so that they were not further separated from the SUVs.

3.3 Characterization of liposomes

The chemical and physical characteristics of liposomes determine their *in vivo* and *in vitro* behaviors. They are extensively used as vehicles for the targeted delivery of drugs. The fate of intravenously injected liposomes is determined by a number of properties. Thus, the quality control of liposomal dispersions is essential. Two of the most important are particle size and zeta potential. Their size is important, as it is one factor that decides about the uptake of the vector into cells. Also for a possible in vivo use, small size has to be ensured to avoid complications like clotting of blood vessels. Surface charge of the particles in dispersion is an important parameter as the potential of liposomes plays a role, for example in stabilizing liposomes against aggregation or fusion and in the interaction between liposomes and charged drugs. It also has an impact on the behaviour of liposomes *in vivo* [Cevc, G. (1993)]. Any subsequent modification of the liposome surface can also be monitored by measurement of the zeta potential. The techniques applied for detecting those parameters are portrayed as follows.

3.3.1 Photon correlation spectroscopy

Particle size, as well as the size distribution of dispersions in the nano-range can be estimated using Photon correlation spectroscopy (PCS), also called Dynamic light scattering (DLS). It is the analysis of the time dependence of intensity fluctuations in scattered laser light (helium, neon or argon) due to the Brownian motion of particles in solution/suspension [Ostrowsky N. (1993)]. Intensity of the stray light fluctuates as the particles in dispersion show Brownian motion. The speed of the Brownian motion corresponds to particle size according to the Stokes-Einstein equation. Small particles diffuse more rapidly than large particles, and the rate of fluctuation of scattered light intensity varies accordingly. Therefore, the hydrodynamic diameter of the particles can be deduced. Results of this calculation include two parameters: the Z-average as mean calculated hydrodynamic diameter describing the size of particles and the Poly-Dispersity Index (PDI) describing the particle size distribution width. PCS is simple and rapid to perform, but misleading results are easy to obtain for heterogeneous systems exhibiting bimodal or more complex size distributions [M.J. Hope, M.B. Bally etc. (1986)].

The size of the liposomes, the size distribution and the zeta-potential of the liposomes were measured in a Malvern Zetasizer Nano series (Malvern Instruments Ltd, Worcestershire, United Kingdom). The data were evaluated with the general purpose analysis model of the Dispersion Technology Software Version 5.02 (Malvern Instruments). The cell type chosen

for Z-average measurement was ZENO112 – Low volume disposable sizing cuvette. This type of cuvette allows an appropriate amount of sample in the range of 400-600µL. For 10 mM liposomes with a Z-average of approximately 150 nm, the samples were prepared as 10 µL sample + 390 µL distilled water. For 50 mM liposomes, the samples were prepared as 50 µL sample + 395 µL distilled water. Unless stated otherwise three measurements at measurement position 4.65 using run time and attenuator (intensity adjustment) around 6-8 recommended by the analysis software were performed.

3.3.2 Zeta-potential measurement

Zeta-potential, defined as the potential difference between the dispersion medium and the stationary layer of fluid attached to the dispersed particle, is determined by applying a voltage to the dispersion and measuring the velocity of the electrophoretic movement of charged particles in the electric field [Müller R.H., (1996)]. The zeta-potential is strongly dependent on the pH of the dispersant and also influenced by its ionic strength.

Zeta-potential of samples was determined after dilution in filtered 10 mM Tris buffer pH 7.4 in a DTS1060-Green disposable zeta cell from Malvern Instruments, (Worcestershire, United Kingdom) on the same Malvern Zetasizer Nano series in three consecutive measurements. The conductivity was controlled to be lower than 1 mS/cm and the attenuator was around 6-8. Zeta-potential was calculated according to the Smoluchowski model using the general purpose analysis model [Müller R.H., (1996)].

3.3.3 Transmission Electron Microscopy

Transmission electron microscopy (TEM) might be another alternative to estimate the lamellarity of liposomes. Three techniques are normally used for the preparation of the samples: Negative staining gives a straight-forward impression of the particle size distribution (provided there are no staining artefacts due to pH, ions, osmoleity), although the lamellarity and morphology of the lipid are difficult to assess. But sample preparation may induce fusion or aggregation of the liposomes. In freeze fracture technique, the hydrophobic monolayer faces are exposed and depicted in detail by the shadowed replicas. These images readily reveal the packing geometries of lamellar and hexagonal phases as well as rippled morphologies. Cryogenic-transmission electron microscopy (Cyro-TEM), finally, is a powerful approach to visualize the three-dimensional geometry and the DNA-load of vesicular structures trapped within a thin layer of ice, even though the contrast is

comparatively low [Anne S. Ulrich, (2002)]. By this technique, direct and detailed information may be obtained with a minimum disturbance of the original sample structure.
In PXL transfer experiment, structures of donor and acceptor liposomes before and after incubation were controlled by TEM, to check if the structure integrity of liposomes was changed.
In the present study, the samples for electron microscopic examinations were carried out by using Cryo-TEM technique: 5 µL of samples were taken on a coated copper grid (Quantifoil R1.2/1.3, Quantifoil, Jena, Germany) and excess of sample sucked off with a piece of filter paper. To avoid the formation of crystals the grid was plunged rapidly into liquid ethane in a cryobox (Carl Zeiss NTS GmbH) and thereby shock-frosted at –175°C. After transfer in frozen state in a Gatan 626 cryotransfer system (Gatan GmbH, München, Germany) into the pre-cooled transmission electron microscope Philips CM 120 (Philips, Eindhoven, NL) the sample was examined at 120 kV.

3.3.4 Differential Scanning Calorimetry

Differential scanning calorimetry (DSC) is a technique for measuring the energy necessary to establish a nearly zero temperature difference between a substance and an inert reference material, since the two specimens are subjected to identical temperature regimes in an environment heated or cooled at a controlled rate. The basic principle underlying this technique is that, when the sample undergoes a physical transformation such as phase transitions, more (or less) heat will flow to it than the reference to maintain both at the same temperature.
Hydrated phospholipids may exist in one or more mesomorphic forms. Many phospholipid vesicles undergo a well-defined transition from gel to liquid crystalline phase, which can be recorded using DSC. Analysis of the phase transitions between these forms is necessary because the state and fluidity of the bilayers is an important parameter of *in vitro* and *in vivo* liposomal stability and drug release profiles. The phase transition temperature (T_m) primarily depends on the vesicle composition. To some degree, it also depends on the vesicle size and curvature [Lichtenberg D. et al., (1981)].
DSC studies were performed in a Perkin-Elmer DSC Pyris 1 (Waltham, MA, USA). Hermetically sealed empty 15-µl-standard aluminum pans were used as reference. 13 µL of liposome suspensions (approximately 9 to 15 mg) were accurately weighed into 15-µL-standard aluminium pans (Perkin Elmer) and heated above the melting temperature (isotropic melt) of the respective liposomal formulations (55 °C for DPPC-liposomes and 65 °C for

DSPC-liposomes) and cooled to 10 °C to crystallize the liposomes and heated up again using a scan rate of 5 °C/min. The calibration of the instrument was carried out by using Milli-Q water and indium. Thermodynamic data were analyzed with Perkin-Elmer DSC Version 7.0 software to determine the peak temperature (T_m) and the enthalpy of the transition of the liposomal mixture (ΔH). Each analysis was repeated three times and considered acceptable if T_m values had coefficients of variation < 10 and 20 % for the ΔH values [Zhao L, et. al., (2003)]. The measurement results of the samples were normalized for comparison.

Chapter 4

Drug transfer experiments

4.1 Preparation of column filling material

CM (carboxy methyl) Sepharose FF (fast flow) is a weak cationic exchange material. It can retain the positively charged donor liposomes and allows the neutral acceptor liposomes to pass through the columns on the basis of differences in their net surface charge. The purchased CM Sepharose FF was pre-equilibrated in a 20 % ethanol buffer solution (served as a bacteriostatic agent), which had to be removed before use. The commercial container of CM Sepharose FF in ethanol was shaken gently to obtain a homogeneous suspension. A total of 50 ml of the gel slurry was transferred into a 200 ml Erlenmeyer flask. The gel material was washed in three steps with Tris buffer at pH 7.4, in the sequence of a decreased ionic strength. The gel slurry was washed for the first time with a 2 times excess of 1 M Tris buffer, pH 7.4. The slurry was shake gently for 40 times. The Erlenmeyer flask was then left on the table for 15 min until the gel settled down. The supernatant buffer was decanted off by means of a Pasteur pipette connected via tube to a water jet vacuum. The gel was washed for the second and third time with a 2 times excess of 150 mM and 10 mM Tris buffer, pH 7.4, and shake gently for 40 times. The buffer was decanted off. In a last washing step, the gel was kept 1:1 (v/v) in 10 mM Tris buffer, pH 7.4, at 4 °C. The gel slurry should be degassed before packing into the columns. DEAE (diethylaminoethyl) Sepharose CL-6B gel, a weak anionic exchange material, could retain the negatively charged donor liposomes and allow only the neutral acceptor liposomes to pass through the columns on the basis of differences in their net surface charge. The preparation of the DEAE Sepharose CL-6B gel was prepared in the same way as the CM Sepharose FF gel.

4.2 Preparation of ion-exchange mini-column

The column is made of Perspex®, 5 cm in length, with an inner diameter of 0.5 cm. The bed column volume is 500µl. Columns, rubber rings and sealing were washed with Milli-Q-water and dried before use. The inner side of the outlet of the column was plugged with around 10 mg glass wool. All material was equilibrated to room temperature, at which the chromatography was performed. The gel was prepared as described in section 4.1 and degassed before filling into micro-columns.

A syringe body of 1 ml, as packing reservoir, was mounted vertically onto the columns through the sealing. 1.0 ml of the treated gel slurry was poured by a Pasteur pipette in a single operation into the packing reservoir. Air was eliminated from the column dead spaces by flushing with distilled water. No air should be trapped inside the column. The Pasteur pipette should be held against the wall of the reservoir when pouring the slurry to minimize the introduction of air bubbles. The reservoir was then filled to the top with distilled water, and connected to a pump system (LKB Pump P-1, Pharmacia, Uppsala, Sweden). The column was eluted with 1.0 ml distilled water (30 drops in 45 s) and packed at the same time. A total of 1.5 ml water (at least two column volumes of the eluent) was applied to equilibrate the column, and the eluent was discarded. A total of 50 µl of saturation liposomes were applied to the columns to reduce non-specific adsorption and improve recovery of acceptor vesicles. This eluent was also discarded. After all these procedures, the columns were ready for use. Directly following this, a 50 µl aliquot of the incubation liposomes was applied to the column, allowed to enter the column completely, and immediately eluted with 1.5 ml of Milli-Q water; elution was complete within 1 min. Possible transfer of drug or label to the column is negligible over 1 min at room temperature.

For each and every application of liposomes, a column with freshly packed gel was used. The regeneration of the used gel was not performed, due to the security reasons of the application of radioactive labelled liposomes in some of the experiments. Sufficient numbers of columns were prepared before the drug transfer experiment. The packed columns were seated at constant room temperature in a big container filled with distilled water to avoid the dehydration of gel. Temperature variation was strictly controlled to avoid the generation of air bubble in the column. The freshly packed columns were consumed on the same day of preparation.

4.3 Experimental designs

Two main experimental parts are included in the study. The first part is about the transfer of paclitaxel, cholesterol and cholesteryl-oleoyl-ether between liposomal membranes. The experiments are based on the *in vitro* ion-exchange micro-column model. The second part is about the transfer of temoporfin between liposomal membranes. These experiments are monitored by fluorescence spectrometer, based on the dequenching of fluorescence (see Section 4.3.2 for explanation).

4.3.1 Drug transfer based on ion-exchange micro-column technique

In this experimental part, transfer kinetics of paclitaxel (PXL), cholesterol and cholesteryl-oleoyl-ether (COE) between liposomal membranes was performed to investigate the possible factors which influence the transfer of drugs between membranes. Cholesterol, and its derivative (COE), are considered as two lipophilic drugs and their transfer kinetics are compared with the transfer kinetics of PXL. Cholesterol is usually considered as a lipid and is often included in liposome formulations to modify the bilayer fluidity. A small amount of cholesterol was therefore incorporated in the cholesterol and cholesteryl-oleoyl-ether containing liposomes to improve the liposome bilayer properties. In PXL containing liposomes, no cholesterol was incorporated in the membrane to avoid the possible influence of cholesterol to the transfer of PXL. The composition of the lipids of positively charged cholesterol or COE containing liposomal formulations was prepared in accordance with PXL containing liposomes. Negatively charged cholesterol and COE containing liposomal formulations were prepared in order to investigate whether the presence of DOTAP influences the transfer kinetics of the drug. Their lipids composition was selected according to a similar study from Fahr A. and co-workers [Fahr A. and Seelig J., (2001)]. The studies have been conducted at room temperature which is considered as the temperature of the physical state that exists in biomembranes. The transfer amount of PXL at different time points was measured by HPLC/UV. Radiolabeled cholesterol and COE were used to mimic the structure of the unmodified molecules in order to avoid the complications arising from addition of reporter groups. The transfer of cholesterol and COE was traced and qualified by radioactivity counting.

Three main steps of experiments were designed and performed according to the development of the *in vitro* ion-exchange micro-column model. The linearity test of the analysis instruments was presented as the first procedure to ensure the accuracy of the LSC (Liquid Scintillation Counter) and HPLC (High Performance Liquid Chromatography). The second

step was to optimize the working capacity of the ion-exchange micro-column. The optimal amount of saturation liposomes for the pre-treatment of the column filling gel, the optimal liposome amount applied on the columns, and the optimal donor acceptor incubation ratio were achieved. As the last step, the transfer of the three selected drugs, paclitaxel, cholesterol and cholesteryl-oleoyl-ether between liposomal membranes was investigated.

4.3.1.1 Linearity tests

The calibration of High Performance Liquid Chromatography (HPLC) was performed from a stock solution containing 1.0 mg paclitaxel/mL ethanol. From the stock solution working solutions were diluted with acetonitrile / tetrahydrofuran / 2 mM ammoniumacetate (48/18/34, v/v/v) to a concentration range from 0.14 to 6.91 µM, with three times repetition at each concentration injection.

The calibration of Liquid Scintillation Counter (LSC) was performed to make sure the accuracy of the three protocols provided by the instrument. Protocol one detects only [^{3}H] radiation, protocol two detects only [^{14}C] radiation, and protocol three detects both [^{3}H] and [^{14}C] radiation at the same time. [^{3}H] labelled donor liposome DOTAP/DOPC/Chol/[^{3}H]Chol in the molar ratio of 25/72/3 and [^{14}C] labelled acceptor liposomes POPC/Chol/[^{14}C] CO with the molar ratio of 97/3 were used in the experiment. Three liposome groups: I) Donor : water = 1 : 5 (v/v), II) Acceptor : water = 1 : 5 (v/v) and III) Donor : Acceptor = 1 : 5 (v/v) were designed for the three protocols respectively. From each incubation group, 0.2 to 50 µL of the suspension were transferred directly into a 20 mL scintillation vial, diluted to 1.5 mL by Milli-Q water, after the addition of 12 mL scintillation cocktail, the mixture was vortexed for 3 minutes until a homogeneous liquid was obtained. For each concentration, only one sample was prepared. The prepared samples were measured by the selected protocols.

For the Calibration of fluorescence spectrometry, the linearity curves of both rhodamine fluorescence and temoporfin fluorescence were determined. The rhodamine standard solutions were prepared by dilution of donor liposomes (DOTAP/DOPC/DOPE-Rho/PXL in a molar ratio of 25/71/1/3) into water to the final rhodamine concentrations between 0.83×10^{-2} and 26.7×10^{-2} µM. The intensity of the standard solutions was measured at E_x = 540 nm and E_m = 585 nm. For each concentration, three repetitions were done. The calibration of temoporfin fluorescence intensity curve was performed by using a specially prepared temoporfin containing liposomes with extremely low temoporfin amount (DPPC / DPPG / TP in the mg ratio 90.0 / 10.0 / 0.2). The temoporfin standard solutions were prepared by dilution of this liposome to a temoporfin concentration range from 10 to 500 ng/mL. The fluorescence

intensity of the working solutions was measured at Ex = 410 nm and Em = 653 nm. For the standard solutions with a temoporfin concentration from 10, 25, 50, 100, 150 to 200 ng/mL, the fluorescence intensity after the addition of 100 µL of 10 % (m/v) Triton X-100 was also measured. The Triton X-100 influence factor was calculated from the difference in fluorescence intensity. The maximal fluorescence intensity of the temoporfin-containing liposomes used in temoporfin transfer study was adjusted by this factor. The calibration of the instruments was performed every time before the measurements. One example of the calibrations from each instrument are presented in section 6.2.

4.3.1.2 Optimization of the ion-exchange micro-column

4.3.1.2.1 Optimal amount of saturation liposomes for the two types of ion-exchange gels

The capacity and reliability of the *in vitro* ion-exchange micro-column model was determined and the optimized parameters were selected for the drug transfer experiment.

An optimal amount of saturation liposomes was necessary for reducing the non-specific adsorption of the gel and improving the recovery of acceptor liposomes. In a previous study, a total of 0.25 mg of sonicated egg yolk phosphatidylcholine vesicles in 0.1 mL of buffer served to this purpose for a 1 cm long column filled with ion-exchange resin [McLean L.R. and Phillips M.C. (1981)]. In order to look for the most suitable amount of saturation liposomes for the ion-exchange columns under the experimental conditions, the columns were first saturated by different amount of saturation liposomes. These pre-saturated columns were used to separate the donor from acceptor liposomes. The optimal saturation liposome amount is evaluated as the columns with the highest separation efficiency. Columns filled with CM Sepharose FF or DEAE Sepharose CL-6B gel were pre-saturated by from 0 to 300 µL saturation liposomes. It was known that the radioactive labeller [^{14}C] CO (Cholesteryl [1-^{14}C] oleate) and [^{3}H] COE ([1α,2α(n)-^{3}H] Cholesteryl-oleoyl-ether) are widely used as a non-exchangeable marker to monitor vesicle recovery [Bar L.K. , et al., (1986); Fahr A and Seelig J., (2001)]. Therefore, the donor and acceptor liposomes are traced with [^{3}H] COE and [^{14}C] CO, respectively. 10 µL of mixture of donor liposomes (DOTAP/DOPC/Chol/[^{3}H]COE in the mole ratio of 25/72/3) and acceptor liposomes (POPC/Chol/[^{14}C] CO in the molar ratio of 97/3) in the volume ratio of 1 to 5 were applied on the CM Sepharose FF gel filled columns, the columns were then eluted immediately with 1.5 mL water. In the case of DEAE Sepharose CL-6B gel filled columns, donor liposomes (DCP/DOPC/Chol/[^{3}H]COE in the molar ratio of 2/7/1) and acceptor liposomes liposomes (POPC/Chol/[^{14}C] CO in the molar ratio of 8/2) were used. The donor and acceptor liposomes have the same lipids concentration of 10 mM.

Three repetitions were performed at each saturation liposome amount. The recovery of donor and acceptor liposomes was measured by Liquid Scintillation Counting (LSC).

4.3.1.2.2 The maximal liposome application amount on the columns

The recommended sample application volume for a sepharose chromatography is 2-5 % of the total bed volume [Handbook from GE Healthcare]. The bed column volume of the ion-exchange micro-columns used in the study was 500 µl. Thus, the optional sample volume applied on the ion-exchange micro-columns should be 10-25 µL. In the study, a 10 µL of liposome application amount is used for the transfer of radioactive compounds, that is to say, for the transfer of cholesterol and cholesteryl-oleoyl-ether from positively or negatively charged donor liposomes. The application amount of 10 µL liposomes is sufficient for being detected by LSC and is within the recommended sample application volume. Due to the limited HPLC/UV detection (a restricted detection limit at 0.3 µg/mL and a quantification limit at 1 µg/mL) for PXL transfer, a higher liposome application amount is required. However, this maximal loading volume should not exceed the capacity of the ion-exchange micro-column. The following experiments were therefore designed to look for the maximal liposome amount that can be applied on the CM Sepharose FF gel filled columns in PXL transfer study.

The acceptor POPC/[^{14}C] CO liposomes used in the experiment were traced by the non-exchangeable label [^{14}C] CO. The donor blank liposomes DOTAP/DOPC/DOPE-Rho in a molar ratio of 25/74/1 were traced by Rhodamine. The donor and acceptor liposomes were quantified by fluorescence measurement and LSC measurement, respectively. Three incubation groups were designed as I) Donor : water = 1 : 1 (v/v), II) Donor (10 mM) : Acceptor (10 mM) = 1 : 1 (v/v) and III) Donor (10 mM) : Acceptor (50 mM) = 1 : 1 (v/v). Immediately after the incubation, 10, 20, 30, 40, 50 and 60 µL of the suspension were applied on the columns and eluted with 1.5 ml water, six repetitions were performed at each liposome application volume. For incubation groups II) and III), the same procedure was also performed 30 min after incubation, in order to investigate if there is an influence of incubation time to donor and acceptor recovery.

4.3.1.2.3 Donor acceptor incubation ratio for CM Sepharose FF gel-filled micro-column

A 10 times of acceptor vesicles over donors was usually preferred for the *in vitro* ion-exchange micro-column experiments [McLean and Phillips (1981); Bar et al., (1986); Fahr and Seelig (2001)]. Due to the limitation of sample application amount on the ion-exchange micro-column in paclitaxel transfer study, it is necessary to increase the acceptor/donor ratio via the enlargement of acceptor lipid concentration instead of the amplification of acceptor application volume. As a result, in the case of paclitaxel transfer study, donor lipids concentration was fixed at 10 mM. Acceptor liposomes were prepared with a lipids concentration of 50 mM, which is maximal 5 times in excess to donor. A higher lipid concentration than 100 mM was not selected, because at this concentration the extrusion was very difficult to carry out, and the polycarbonate membrane got easily broken induced from the high lipids concentration. The purpose of this experiment is to investigate whether the change in acceptor lipids concentration influences the separation of the donor from acceptor liposomes by ion-exchange micro-columns in the paclitaxel transfer study. The donor acceptor incubation ratio used in the transfer of cholesterol and cholesteryl-oleoyl-ether study will be in accordance with the ratio in paclitaxel transfer study.

Three donor blank liposomes were used: 1) DOTAP/DOPC/DOPE-Rho in a molar ratio of 5/94/1, 2) DOTAP/DOPC/DOPE-Rho in a molar ratio 25/74/1 and 3) DOTAP/DOPC/DOPE-Rho/DOPE-PEG in a molar ratio of 25/69/1/5, lipid concentration of donor was 10 mM and acceptor POPC/[^{14}C] CO liposome with the lipid concentration at 50 mM. Acceptor liposomes at lower lipids concentrations were obtained by dilution of 50 mM acceptor liposomes. Three incubation groups were designed: I) Donor (10 mM) : water = 1 : 1 (v/v), II) Acceptor (10, 20, 30, 40 and 50 mM): water = 1 : 1 (v/v) and III) Donor(10 mM) : Acceptor (10, 20, 30, 40 and 50 mM) = 1 : 1 (v/v). A similar procedure was performed for each incubation group, i.e. 30 minutes after incubation, 50 µL of the suspension from each incubation group was applied on the columns and eluted with 1.5 mL water. Six repetitions were performed for each incubation group.

The recovery of donor liposomes was measured by fluorescence spectroscopy and the recovery of acceptor liposomes was counted by LSC. The analytical methods are described in Section 5.1, 5.2 and 5.3.

4.3.1.3 Paclitaxel transfer between liposomal membranes

In the commercially available liposomal formulation EndoTAG^{TM}-1 produced by MediGene, paclitaxel in incorporated in a positively charged liposomes. It is of great interest to investigate the transfer kinetics of paclitaxel *in vivo*. Based on this requirement and due to the complexity of the *in vivo* study, the *in vitro* ion-exchange micro-column model was designed in order to look for the possible paclitaxel transfer kinetics *in vitro*. It is known that the use of liposomes as vehicles for drug delivery is limited because of the short survival time of plain liposomes in blood. The incorporation of a well-balanced amount of polymer lipids can effectively extend the circulation time of the liposomes *in vivo*. Based on this awareness, the long circulating paclitaxel-containing liposomes prepared by phospholipids with covalently attached PEG lipids were also prepared, in order to investigate the influence of PEG on the transfer of paclitaxel between liposomal membranes. The two paclitaxel-containing liposomal formulations used in this section are DOTAP/DOPC/DOPE-Rho/PXL in a molar ratio of 25/71/1/3 and DOTAP/DOPC/DOPE-Rho/DOPE-PEG/PXL in a molar ratio of 25/66/1/5/3.

4.3.1.3.1 Linear relation of paclitaxel transfer

The water solubility of paclitaxel has been reported to be 0.50 ± 0.05 µM [Wenk M.R. et al., (1996)]. This means that there is always the equilibrium of 0.5 µM paclitaxel dissolved in water. Presuming an equal distribution of paclitaxel between donor and acceptor liposomes, when the paclitaxel-containing donor liposomes are incubated with increased amount of blank acceptor liposomes, a linear relation should be obtained between the concentration of paclitaxel transferred to acceptor liposomes with the ratio of acceptor lipid concentration to the summation of donor and acceptor lipid concentrations ([PXL] ∝ [Acc.]/[Don.]+[Acc.]). According to these assumptions, the experiments were designed in order to check the linearity of paclitaxel transfer at different acceptor lipids concentrations.

Two types of paclitaxel-containing donor liposomes were prepared as DOTAP/DOPC/DOPE-Rho/PXL in a molar ratio of 25/71/1/3 and DOTAP/DOPC/DOPE-Rho/DOPE-PEG/PXL in a molar ratio of 25/66/1/5/3, both at a constant lipid concentration of 10 mM. The POPC liposomes with lipids concentration of 50 mM were used as acceptor liposomes. In order to quantify the acceptor recovery, Cholesteryl [1-^{14}C] oleate labelled radioactive POPC/[^{14}C] CO liposomes were prepared. Acceptor liposomes at lower lipid concentrations were obtained by dilution from acceptor liposomes of 50 mM. Three incubation groups were designed as I) Donor (10 mM) : water = 1 : 1 (v/v), II) Acceptor (10, 20, 30, 40 and 50 mM) : water = 1 : 1

(v/v) and III) Donor (10 mM) : Acceptor (10, 20, 30, 40 and 50 mM) = 1 : 1 (v/v). After 30 min incubation, 50µL liposome suspensions from all the incubation groups were applied on the columns and eluted by 1.5 ml water. Three repetitions were performed for each incubation group.

The recovery of donor liposomes was measured by rhodamine fluorescence spectroscopy. The recovery of radioactive acceptor liposomes was measured by LSC. The recovery of non-radioactive acceptor liposomes should be in agreement with the recovery of radioactive acceptor liposomes since the incubation of donor with non-radioactive acceptor liposomes and the incubation of donor with radioactive acceptor liposomes were designed in an identical way. Similarly, only the quantification of paclitaxel transfer from the donor with non-radioactive acceptor liposomes incubation was measured by HPLC. The transfer of paclitaxel from donor to radioactive acceptor liposomes was not measured due to the strict security issues of the radioactive substances by HPLC measurement.

4.3.1.3.2 Paclitaxel transfer kinetics from two types of donor to acceptor liposomes

The transfer kinetics of paclitaxel between liposomal membranes during the incubation was measured as the transfer amount of paclitaxel from donor to acceptor liposomes at selected time points during 30 min incubation. Two donor liposomal formulations were used: DOTAP/DOPC/DOPE-Rho/PXL in the molar ratio of 25/71/1/3 and DOTAP/DOPC/DOPE-Rho/DOPE-PEG/PXL in the molar ratio of 25/66/1/5/3 (10 mM lipid concentration). Both non-radioactive POPC liposomes and Cholesteryl [1-^{14}C] oleate labelled radioactive POPC/ [^{14}C] CO liposomes were used as acceptor liposomes. The lipids concentration of acceptor liposomes was 50 mM. Two incubation groups were designed: I) Donor:Water = 1 : 1 (v/v) and II) Donor (10 mM):Acceptor (50 mM) = 1 : 1 (v/v). At the selected time points 0.5 min, 2 min, 5 min, 10 min, 15 min, 30 min, 50 µl of the suspensions were applied on the columns and eluted by 1.5 ml water, three repetitions at each time point.

As explained in Section 4.3.1.3.1, the incubation of donor with radioactive acceptor liposomes was measured to evaluate the recovery of acceptor liposomes. The recovery of non-radioactive acceptor liposomes was not measured, and was considered to be similar as the one of radioactive acceptor liposomes. The liposomes used for HPLC paclitaxel quantification measurement were not radioactive.

4.3.1.4 Cholesterol transfer between liposomal membranes

Cholesterol transfer from both positively and negatively charged donor liposomes to acceptor liposomes was studied. Positively charged donor cholesterol-containing liposomes were composed of DOTAP/DOPC/Chol/[^{3}H]Chol in the molar ratio of 25/72/3. The lipid composition of positive donor cholesterol-containing liposomes was in the same ratio as positive paclitaxel-containing liposomes. The negatively charged cholesterol-containing liposomes were composed of DCP/POPC/Chol/[^{3}H]Chol in the molar ratio of 1/7/2. This composition ratio was selected according to a similar experimental design from Fahr A. and co-workers [Fahr A. and Seelig J., (2001)]. The lipid concentration for both positive and negative cholesterol-containing donor liposomes was 10 mM. The cholesterol used for transfer purpose was labelled with [^{3}H] Chol ([1α,2α(n)-^{3}H]Cholesterol) to a final concentration of 1 µCi/mL liposomal suspension. Acceptor liposomes were composed of POPC/Chol/[^{14}C] CO in a molar ratio of 97/3 for incubation with positively charged donor liposomes and POPC/Chol/[^{14}C] Co in a molar ratio of 8/2 for incubation with negatively charged donor liposomes. The acceptor lipid concentration was 10 mM. The lipids of acceptor liposomes were traced with [^{14}C] CO (Cholesteryl [1-^{14}C] oleate). Three incubation groups were designed as I) Donor:Water = 1:5 (v/v), II) Water:Acceptor = 1:5 (v/v) and III) Donor:Acceptor = 1:5 (v/v). At time points of 2 min, 5 min, 10 min, 15 min, 30 min, 60 min, 120 min, 240 min and 2440 min, 10µL of the suspension from each incubation group was applied on the columns and eluted by 1.5 ml water, three repetitions were performed at each time point.

Donor and acceptor vesicles had the same lipid concentration and were incubated in the volume ratio of 1 to 5, in order to be in agreement with the transfer experiments of paclitaxel, where donor was incubated with the same volume of 5 times more concentrated acceptor. Due to the high sensitivity of LSC, the application amount of only 10 µL liposomal suspension on the columns was enough to be detected. The transfer of cholesterol between liposomal membranes during 24-hour incubation was quantified by [^{3}H] measurement and the recovery of acceptor liposomes was controlled by [^{14}C] measurement.

4.3.1.5 Cholesteryl-oleoyl-ether transfer between liposomal membranes

The transfer of the derivative of cholesterol, cholesteryl-oleoyl-ether (COE) from the COE-containing liposomal formulations was also studied as a comparison for the transfer of paclitaxel and cholesterol. Similar as the composition of cholesterol-containing liposomes, positively and negatively charged COE containing liposomes were prepared as

DOTAP/DOPC/Chol/[^{3}H] COE in the molar ratio of 25/72/3 and DCP/POPC//Chol[^{3}H] COE in the molar ratio of 2/7/1. COE was labelled with [^{3}H] in a final radioactivity of 1 µCi/ mL liposome suspension. Acceptor liposomes for incubation with positively charged donor liposomes were composed of POPC/Chol/[^{14}C] CO in a molar ratio of 97/3, and the acceptor liopsomes for incubation with negatively charged donor liposomes were composed of POPC/Chol/[^{14}C] Co in a molar ratio of 8/2. The lipid concentration of both donor and acceptor liposomes was 10 mM. The lipids of acceptor liposomes were traced with [^{14}C] CO (Cholesteryl [1-^{14}C] oleate). The experiments were designed in the same way as in cholesterol transfer experiments. The transfer of COE between liposomal membranes during 24 hours incubation was quantified by [^{3}H] measurement and the recovery of acceptor liposomes was controlled by [^{14}C] measurement.

4.3.2 Temoporfin transfer based on fluorescence dequenching effect

On the basis of the fluorescent character of temoporfin, the transfer (or the release) of temoporfin from donor liposomes can be estimated by the increase of fluorescence intensity at different time points in the *in vitro* drug transfer experiment. The fluorescent substance shows a self-extinguishing effect of the fluorescence at a higher concentration. This phenomenon is called the self-quenching effect. This effect is due to the non-increasing behaviour of the fluorescence intensity at a high concentration, then reaching a plateau or even showing a decrease [Kellner et al., (2004)]. Temoporfin shows an evident self-quenching effect even at quite low concentration, and the release of temoporfin gives rise to enhanced fluorescence intensity. This character is proved to be the easiest and fastest method of demonstrating the kinetics transfer of temoporfin from donor to acceptor liposomes. In the presented study, two types of lipids, i.e. DPPC/DPPG and DSPC/DSPG, based temoporfin liposomal formulations were prepared. In order to simplify the names of the liposomal formulations, the abbreviation of the component was used. DPPC/DPPG is named as C16, DSPC/DSPG is replaced by C18, temoporfin is substituted by TP, DPPE-mPEG 2000 (used in DPPC/DPPG based formulations) and DSPE-mPEG 2000 (used in DSPC/DSPG based formulations) are shorten to P, and cholesterol is named Chol. The lipid compositions of all the liposomal formulations are given as follows: 1) C16, 2) C16_TP, 3) C16_TP_Chol, 4) C16_TP_P, 5) C16_TP_P_Chol, 6) C16_ DOTAP_TP, 7) C18, 8) C18_TP, 9) C18_TP_Chol, 10) C18_TP_P and 11) C18_TP_P_Chol (see Table 3.2 for composition ratios). The size and PDI data, the phase transition of the all these eleven liposomal formulations and the transfer of

temoporfin between liposomal membranes from eight selected temoporfin-containing liposomal formulations (except C16_DOTAP_TP formulation) will be described in Chapter 7. The transfer of temoporfin from different donor liposomal formulations to acceptor liposomes was examined with the purpose to study the influence of different lipid components to the transfer behaviour of temoporfin at two selected temperatures. Two incubation temperatures, 25 and 37 °C, were selected. 25 °C is widely used as the temperature for physical models, and 37 °C is the normal body temperature.

From all the eleven donor liposomal formulations, eight temoporfin-containing donor liposomal formulations were selected for the temoporfin transfer experiment. The temoporfin contain of these liposomal formulations was 1.50 ± 0.05 mg/ml, quantified by UV absorbance. These eight liposomal formulations are named as 1) C16_TP, 2) C16_TP_P, 3) DPPC/DPPG_TP_Chol, 4) C16_TP_P_Chol, 5) C18_TP, 6) C18_TP_P, 7) C18_TP_Chol and 8) C18_TP_P_Chol. Temoporfin was incorporated in the bilayer of donor liposomes. The fluorescence of temoporfin was in a self-quenching state. Acceptor liposomes were prepared by POPC lipid as blank liposomes. For acceptor liposomes, the lipids are at a fluid liquid state at the selected two temperatures. The lipid concentration of both donor and acceptor liposomes was 20 mg/mL. Donor temoporfin-containing liposomes were first diluted in a 1 : 10 (v/v) ratio by 5 % glucose solution, from which 3 µL suspensions (i.e. 150 ng/ml TP) were mixed with 30 µl Acceptor liposomes and incubated in Tris buffer (10 mM, pH 7.4) to a final 3 ml in a 10 mm path length quartz cuvette. The suspension was stirred by a magnetic stirrer along the whole incubation time. The transfer of temoporfin fom donor to acceptor was monitored by fluorescence changes using a fluorescence spectrometer. Three repetitions for each incubation group were performed.

Chapter 5

Measurement of drug transfer

5.1 Fluorescence Spectroscopy

Fluorescence spectroscopy is a type of electromagnetic spectroscopy which analyses fluorescence from a sample. It is performed by using a beam of light, usually ultraviolet light, that excites the electrons in molecules of certain compounds and causes them to emit light of a lower energy, typically, but not necessarily visible light. A complementary technique is Absorption Spectroscopy. A fluorescent molecule can be irradiated with different wavelengths within its excitation spectrum and, accordingly, will emit light with a characteristic emission spectrum. Its amplitude is determined by the intensity of radiation and the excitation efficiency, which is a function of the excitation wavelength.

Fluorescence spectra were monitored on a computer-controlled Fluorescence Spectrometer Luminescence Spectrometer LS 50B from Perkin-Elmer (Waltham, MA, USA) with the software program FL WinLab at room temperature. The rhodamine containing elutions from PXL transfer experiments were excited at 540 nm, and the emission was recorded at 585 nm. Both excitation and emission slits were set at 10 nm widths. The emission intensity was recorded 3 times with a response time of 1 s. The integration time was 1 s. Rho was presented in an unquenched state in donor liposomes. The addition of suitable amount of Triton X did not give any difference to the rhodamine intensity while a decreased rhodamine intensity was noticed by the excessive addition of Triton X. Therefore no Triton X was used to lyse the liposomes.

Kinetics of Temoporfin redistribution was monitored by the same fluorescence spectrometer with the sample compartment thermo-stated by Julabo F25-ME thermostat, (Seelbach,

Germany) to the required temperatures. The fluorescence intensity was monitored continuously at a fixed wavelength of emission (λ_{ex} = 410 nm, λ_{em} = 653 nm, band passes for both excitation and emission slits were 10 nm). Data were collected at the selected time points during the incubation of 4.5 hours. At each time point, the signal was integrated for 1 s. At the end of the measurement, the vesicles were disrupted by adding 100 µL of Triton X-100. The resulting fluorescence level was set to 100% and indicated as I_∞. The maximal fluorescence intensity was adjusted by a factor of 1.1 (see section 6.2.3.2 for explanation). The transfer of temoporfin was expressed as percentage of the maximum fluorescence and was calculated from the ratio of $(I_t-I_0)/I_\infty$, where I_0, I_t and I_∞ represent the fluorescence intensity of the incubation solution at times zero, t and ∞, respectively.

5.2 Liquid Scintillation Counting

Liquid Scintillation Counting (LSC) is based on the measurement of the radiation of substances labelled with a radioactive nuclide. The most commonly used beta emitting isotopes are ^{3}H, ^{14}C and ^{32}P. Scintillation cocktail consists of a solvent and scintillator (Fluor). In the scintillation process, beta particles excite molecules of the solvent and from these the energy is transferred to molecules of the scintillator and re-emitted as light. The light is converted to photoelectrons at the photomultiplier photocathode and the resulting pulse is amplified and counted at the detector. Since the emitted light does not always match with the characteristics of the photomultiplier tube (PMT), a secondary scintillator is used. This secondary scintillator absorbs photons of one wavelength and shifts the wavelength to be suitable for the response area of the PMT. A LSC can measure very small amounts of radioactivity with quite high accuracy. The count rate is proportional to the amount of labelled compound or the activity contained in the sample. The number of disintegrations per minute (DPM) of each sample was counted [Pekka Mäkinen, (2001)].

The samples for radioactivity measurement were treated in a standardised way. 1.5 ml of sample eluents was mixed with 12 ml scintillation fluid Rotiszint eco plus (for hydrophilic and hydrophobic samples) in a 20 ml Polyethylene Vials matched with screw caps from Kartell S.p.A. (Noviglio, MI, Italy). The mixture was vortexed for 1 min by Vortex Genie 2 TM, Model G-560E, Scientific industries, INC. (Bohemia, NY, USA). The mixture has to be homogeneous. The presence of phase separation is an indication of unsuccessful treatment. The treated samples were stored at 4 °C and prevented from light for 24 h before measured by a Liquid Scintillation Analyzer Tri-Carb 2800 TR, Perkin Elmer (Waltham, MA, USA). The software program was Quantasmart (TM) Version 2.03. No separation of the phases was

presented during the whole measure process. The protocols for counting the radiation of [^{3}H] or [^{14}C] separately and for counting the radiation of [^{3}H] and [^{14}C] at the same time were used for the measurement. The counting time for each sample was 15 min. In the transfer of cholesterol from [^{3}H]Chol labeled cholesterol-containing donor liposomes to [14C] CO labeled acceptor liposomes, or the transfer of cholesteryl-oleoyl-ether (COE) from [^{3}H] COE labeled COE-containing donor liposomes to [14C] CO labeled acceptor liposomes experiments, the fraction of the label that transferred into acceptor liposomes at time t is given by $X_t = (^3H / ^{14}C)_t / (^3H / ^{14}C)_{mix}$, where $(^3H / ^{14}C)_t$ and $(^3H / ^{14}C)_{mix}$ represent the ratio of [^{3}H] Chol (or [^{3}H] COE) to [^{14}C] CO in the eluent at time t and in the incubation mixture, respectively.

5.3 High Performance Liquid Chromatography

The PXL concentration in the liposomes was determined by HPLC. The samples were diluted by three-time excessive of acetonitrle / tetrahydrofuran / 2 mM ammoniumacetate (48/18/34, v/v/v), the amount of organic solvent was enough to destroy the liposomes. Paclitaxel concentration was determined by HPLC analysis using a well developed method. The HPLC system was equipped with a Agilent 100 Series HPLC System from Agilent Technologies (Palo Alto, CA, USA) consisting of a quaternary pump, online degasser, auto sampler, column thermostat and variable wavelength detector. The mobile phase consisted of acetonitrle / tetrahydrofuran / 2 mM ammoniumacetate (pH-value adjusted to 4.8 with acetic acid) at the ratio of 32/12/56 (v/v/v). The flow rate was 1 ml/min and UV absorbance detection was accomplished at 229 nm. A reverse phase Column LiChroCART 250-4, LiChrospher 60, PR-select B; 250 mm x 4 mm, 5 µm, from Merck (Darmstadt, Germany) was kept in a thermostat at 35 °C. The injection volume was 40 µL. The analysis duration was 40 min for each measurement. The retention time of paclitaxel was about 16 min. The HPLC-method was checked on its ability to investigate possible degradation of paclitaxel during the experiments. The main degradation product of paclitaxel, 7-epi-Paclitaxel, can be detected at the retention time about 25 min. Additionally, 10-deacetyl-7-epi-paclitaxel could also be detected. The HPLC system thus seemed suitable for stability-indicating measurements. No peak deformations of shoulders, which could indicate the presence of a degradation product under the paclitaxel peak, were observed.

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Part III

RESULTS AND DISCUSSION

Chapter 6

Drug transfer based on ion-exchange micro-column technique

6.1 Characterization of liposomes

The Z-average, PDI and zeta-potential of all the liposomal formulations were checked by PCS immediately after liposome preparation as a quality control. Transmission Electron Microscopy of the liposomes in PXL transfer experiments before and after the incubation was performed in order to follow the liposome structure.

6.1.1 Size and surface potential measurements

All the liposomes were freshly self-prepared before the experiments, except the paclitaxel-containing donor liposomes. The lipid thin-films of paclitaxel-containing liposomes were provided by MediGene AG (Martinsried, Germany), and were further hydrated in the lab just before the experiment. The present work has been carried out under cooperation with MediGene AG and the composition of the paclitaxel-containing liposomes was prepared in accordance with the commercially available liposomal formulation EndoTAG^{TM}-1 produced by MediGene. The product of EndoTAG^{TM}-1 is positively charged and the effective component of this product is Paclitaxel$^{®}$, which is one of the most effective substances in chemotherapy. The positive charge of the liposomes enables them to attach themselves selectively to the negatively charged, newly developing endothelial tumour cells (neovascular targeting) and to destroy them (vascular disrupting). This process is intended to suppress nutrient supply and inhibit further tumour growth. The positive charge of the liposomes is

obtained by incorporation of DOTAP into the lipids. Table 6.1 gathers the composition ratios, zeta-potential, average size (Z-average) and PDI for all the liposomal formulations used.

Table 6.1: *Formulation, composition ratios (in molar), Zeta-potential (Z_p), average liposome size and PDI of the liposomes for the ion-exchange micro-column model based drug transfer experiments. All the measurements were performed within the next 24 h after the preparation of the liposomes and all the values were determined from three measurements.*

Liposome type	Formulation	Composition	Z_p (mV)	Z-average (nm)	PDI
Don. - PXL	DOTAP/DOPC/DOPE-Rho/PXL	25/71/1/3	+61.4 ± 5	146.2 ± 5	0.072 ±0.01
	DOTAP/DOPC/DOPE-Rho/DOPE-PEG	25/69/1/5	+32.5 ± 4	186.3 ± 7	0.151 ± 0.03
	DOTAP/DOPC/DOPE-Rho/DOPE-PEG/PXL	25/66/1/5/3	+24.8 ± 4	151,3± 7	0.079 ± 0.02
	DOTAP/DOPC/DOPE-Rho	25/74/1	+65.1 ± 6	150.2 ± 4	0.109 ± 0.01
	DOTAP/DOPC/DOPE-Rho	20/79/1	+58.6 ± 6	147.5 ± 4	0.113 ± 0.01
	DOTAP/DOPC/DOPE-Rho	10/89/1	+52.8 ± 7	148.1 ± 5	0.125 ± 0.01
	DOTAP/DOPC/DOPE-Rho	5/94/1	+26.6 ± 6	144.5 ± 5	0.118 ± 0.02
Don. - Chol	DOTAP/DOPC/Chol/[^{3}H] Chol	25/72/3	+44.7 ± 5	144.9 ± 6	0.099 ± 0.01
	DCP/DOPC/ Chol/[^{3}H]Chol	1/7/2	-56.3 ± 6	148.6 ± 5	0.109 ± 0.01
Don. - COE	DOTAP/DOPC/Chol/[^{3}H] COE	25/72/3	+46.3 ± 3	147.8 ± 4	0.092 ± 0.01
	DCP/DOPC/ Chol/[^{3}H]COE	1/7/2	-50.9 ± 4	146.3 ± 5	0.103 ± 0.01
Acc. - PXL	POPC/[^{3}H]COE	-	-3.98 ± 2	180.5 ± 6	0.272 ± 0.03
	POPC	-	-4.19 ± 2	176.8 ± 7	0.284 ± 0.02
Acc. - positive Chol, COE	POPC/Chol/[^{14}C] CO	97/3	-5.87 ± 3	153.9 ± 7	0.110 ± 0.01
Acc. - negative Chol, COE	POPC/Chol/[^{14}C] CO	8/2	-2.41 ± 1	148.7 ± 5	0.109 ± 0.02
Satu. - PXL	POPC	50	+2.87 ± 1	39.2* ± 7	0.371 ± 0.03
Satu. - positive Chol, COE	POPC/Chol	97/3	-3.15 ± 2	43.5* ± 8	0.412 ± 0.04
Satu. - negative Chol, COE	POPC/Chol	8/2	-3.74 ± 3	41.7* ± 7	0.426± 0.03

**In the size distribution report by intensity, two peaks were observed, one peak at the size distribution of around 40 nm with an intensity of 40 % to 50 %, another peak at the size distribution of 150 nm with an intensity of 50 % to 60 %. The values of the peak with smaller size distribution are indicated in the table.*

From the data shown in Table 6.1, it can be observed: when DOTAP concentration is increased from 10 mol % to 25 mol % in DOTAP/DOPC/DOPE-Rho/DOPE-PEG/PXL formulations, the zeta-potential of the liposomes fluctuate in a small range from +52 to +65 mV. When DOTAP concentration reduces to 5 mol %, the zeta-potential of the liposomes decreases dramatically to only +26.6 mV. The percentage of DOTAP has a fundamental influence on the zeta-potential of the liposomes. A concentration of DOTAP higher than 10 mol % is required in order to ensure a sufficient positive surface charge. As reported in the previous literature, a DOTAP concentration of above 20 mol % in the liposome bilayer is of potential interest for drug delivery study due to high charge densities may be required for optimal cell interaction or incorporation of substances for encapsulation [Felgner P.L., et al., (1987); Felgner P.L and Ringold G.M., (1989)]. According to the relation between zeta-potential and DOTAP concentration for blank donor liposomes, the DOTAP concentration of 25 mol % is selected for the positively charged paclitaxel-containing liposomes. The drug-containing formulation DOTAP/DOPC/DOPE-Rho/PXL (25/71/1/3, molar ratio) has a zeta-potential of +61.4 mV. Compared to the corresponding blank donor formulation DOTAP/DOPC/DOPE-Rho (25/74/1, molar ratio), which has a zeta-potential of +65.1 mV, the incorporation of the drug paclitaxel does not produce a significant influence on the zeta-potential.

However, when polyethyleneglycol (PEG) is incorporated into these two liposomes, the zeta-potential of PEG-containing formulations DOTAP/DOPC/DOPE-Rho/DOPE-PEG-PXL(25/66/1/5/3, molar ratio) and DOTAP/DOPC/DOPE-Rho/DOPE-PEG (25/69/1/5, molar ratio) decreases down to + 24.8 and + 32.5 mV, respectively. The zeta-potential of DOTAP/DOPC/DOPE-Rho/DOPE-PEG-PXL (25/66/1/5/3, molar ratio) is too low, and the positive charge might be insufficient for an effective separation by ion-exchange micro-column. The incorporation of the amphiphilic PEG derivative phospholipids into lipid bilayer gives rise to a noticeable reduction or mask of positive zeta-potential. The decrease in the zeta potential as the surface of the liposome is covered can be explained through the big PEG head groups that are settled outside the lipids. The presence of the PEG chains on the liposome surface reduced the mobility of the liposomes and hence the zeta-potential.

The positively charged cholesterol-containing and cholesteryl-oleoyl-ether (COE)-containing donor liposomes are prepared in the same lipid composition ratio as paclitaxel-containing liposomes. The zeta-potential of these two donor liposomes is around + 45 mV. The negatively charged cholesterol-containing and COE-containing liposomes have zeta-potentials of –56.3 and –50.9 mV. All the acceptor and saturation liposomes should be

neutral, and they possess a zeta-potential in the range from –5.87 to 2.87 mV. The zeta-potential of all the drug-containing donor liposomes, acceptor liposomes and saturation liposomes meets the standards for a successful donor acceptor separation required by the ion-exchange micro-columns, except the formulation with the composition DOTAP/DOPC/DOPE-Rho/DOPE-PEG-PXL(25/66/1/5/3, molar ratio), whose zeta-potential might be lower than the minimal requirement.

The Z-average of all the donor liposomal formulations is around 150 nm with a PDI of around 0.1. The size of POPC acceptor liposomes with a lipid concentration of 50 mM is around 180 nm, and the PDI is more than 0.2. The difficulty during extrusion due to the high lipids concentration is perhaps the reason of the relatively poor size distribution. All the liposomal formulations meet the requirements of liposome quality control.

The Z-average and PDI of donor and acceptor liposomes after separation by ion-exchange micro-column were also measured by PCS. The Z-average of all the liposomes is quite comparable before and after the separation by the ion-exchange micro-column, but the PDI values of the liposomes are a slightly increased after the separation (detailed data are not shown).

6.1.2 Transmission Electron Microscopy

Under the conditions of the ion-exchange micro-column model, the vesicles are incubated in the absence of proteins and they should be stable to fusion over the course of the experiment. In order to exclude fusion from the whole incubation course in the case of paclitaxel transfer, the Transmission Electron Microscopy (TEM) examination of positively charged PXL-donor liposomes and neutral acceptor liposomes before and after 30 min incubation at room temperature was carried out. The Cryo-TEM pictures of donor (DOTAP/DOPC/DOPE-Rho/PXL = 25/71/1/3 molar ratio, 10 mM lipid concentration) and POPC acceptor liposomes (50 mM lipids concentration) used for the study of PXL transfer kinetics experiment are illustrated in the Figure 6.1.

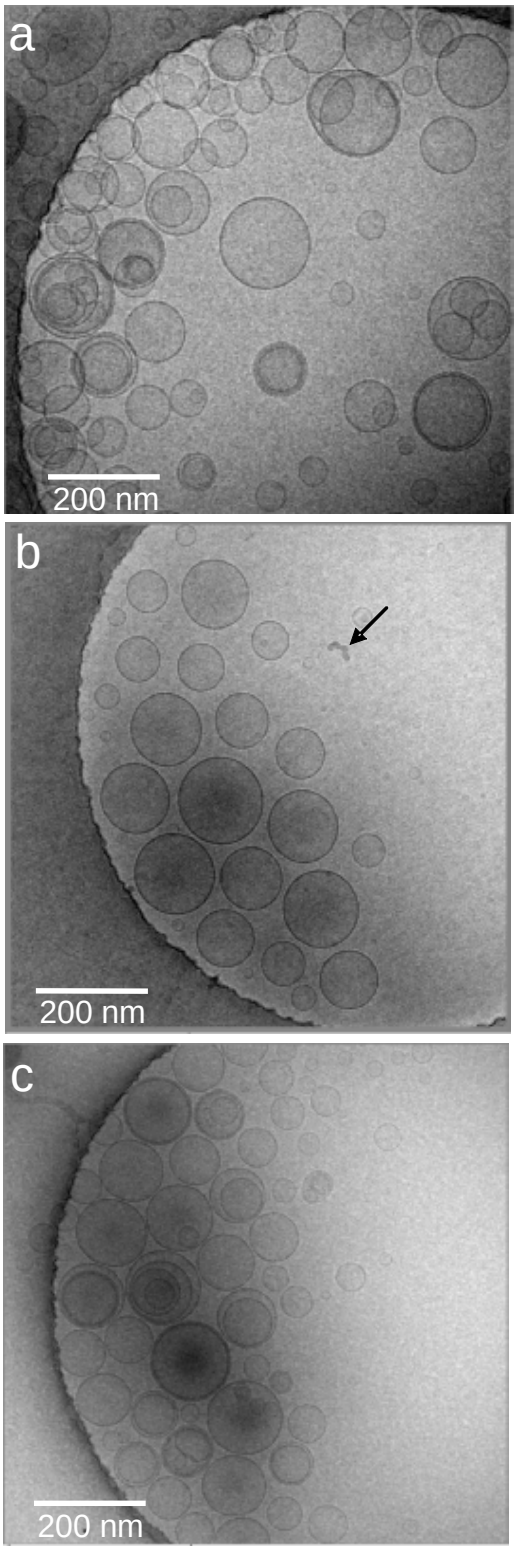

Figure 6.1: *TEM microphotographs of the donor and acceptor liposomes before and after incubation. Acceptor liposomes (lipids composition POPC, lipids conc. 50 mM) before incubated with donor liposomes (a); donor liposomes (lipids composition DOPC/DOTAP/Rho-DOPE/PXL = 71/25/1/3, lipids conc. 10 mM) before incubated with acceptor liposomes (b); incubation mixture of donor and acceptor in a ratio of 1:1(v/v) after 30 min incubation (c).*

Before the incubation, the TEM microphotograph of the donor liposomes (Figure 6.1b) shows unilamellar liposomes with a size distribution in the range from 100 nm to 200 nm. The arrow presented in Fig. 6.1b denotes an ice crystal deposited on the sample surface after vitrification. In the TEM microphotograph of acceptor liposomes (Figure 6.1a), a co-existence of multilamellar, bilamellar and unilamellar liposomes is noticeable, and the size of the vesicles is more variable. This is in accordance with the poor PDI value in PCS measurement. Figure 6.1c shows the TEM microphotograph of donor and acceptor mixture after 30 min incubation. Both donor and acceptor vesicles remain unchanged in TEM pictures. Assuming that the size of the donor and acceptor liposomes is 160 nm, if there is the fusion between donor and acceptor, the size after fusion should be 226 nm, and the shape of the fused liposomes should not be in an intact round shape. As indicated in the TEM picture, all the vesicles remain intact before and after 30 min incubation and no size grow of the liposomes is observed.

The work of Haran N. and co-workers has ruled out fusion as a prerequisite for cholesterol exchange by using Nuclear Magnetic Resonance experiments [Haran N. and Shporer M., (1977)]. This finding is further confirmed as the extent of vesicle-vesicle fusion is negligible over a period of at least 12 h by McLean L.R. and co-workers [McLean L.R. and Phillips M.C., (1981)]. Thanks to the previous work of the exclusion of fusion in cholesterol transfer, the TEM examination of cholesterol- or COE-containing donor liposomes with acceptor liposomes is not performed.

With the proof of the TEM microphotographs of PXL-containing liposomes and the acceptor liposomes, the PSC measurement of the donor and acceptor liposomes before and after separation by ion-exchange micro-columns, together with the no-fusion conclusion during the incubation of cholesterol- and COE-containing liposomes with acceptor liposomes, the ion-exchange micro-column model can be considered as a reliable method to separate successfully the neutral acceptor from charged donor liposomes in an unchanged and intact form.

6.2 Linearity test of analytical instruments

The calibration of all the analytical instruments was carried out every time before the measurements. One of the calibration curves from each analytical device is selected as an example and presented below:

6.2.1 Linearity test of High Performance Liquid Chromatography

The HPLC linearity test was performed by measuring the PXL standard solutions in the concentration range from 0.14 to 6.91 µM. The peak area at each concentration is presented in Table 6.2.

Table 6.2: *Peak areas of PXL standard solutions in the concentration range from 0.14 to 6.91 µM measured by HPLC. Three injections are performed for each concentration. The data are presented as average ± RSD, and are calculated from three repetitions.*

[PXL] (µM)	Peak area
0.14	10.77 ± 0.34 %
0.55	39.87 ± 0.25 %
1.38	97.51 ± 0.41 %
2.76	191.63 ± 0.47 %
6.91	533.53 ± 0.56 %

The peak area presents a linear behaviour as a function of paclitaxel concentration between 0.14 and 6.91 µM (Peak area=77.37*c-6.99, r^2 = 0.9991). In this study, the maximal theoretical PXL concentration in the liposomes is 5 µM, which is within the calibration range. The repeatability of the injection of two paclitaxel standards is in agreement with the Pharmacopoeia criteria from European Pharmacopoeia 2005, Chapter 2.2.46.

6.2.2 Linearity test of Liquid Scintillation Counting

Three different protocols for measuring the radiation of [^{3}H] and [^{14}C] were examined in the LSC linearity test. The samples prepared from incubation mixture of donor liposomes (DOTAP/DOPC/Chol/[^{3}H]Chol in the molar ratio of 25/72/3) and water was measured by protocol one, which counts only the [^{3}H] radiation. The samples prepared from incubation mixture of acceptor liposomes (POPC/Chol/[^{14}C] CO in the molar ratio of 97/3) and water was measured by protocol two, which counts only the [^{14}C] radiation. The samples prepared from incubation mixture of donor and acceptor liposomes was measured by protocol three, which counts both the [^{3}H] and [14C] radiation. Only one sample was prepared for each concentration and no repetition was necessary. The Disintegrations Per Minute (DPM) of radioactive nuclide [^{3}H] or [^{14}C] from each sample measured by different protocols and the coefficient of regression of the least squares analysis of the calibration curves are shown in Table 6.3.

Table 6.3: *DPM results of each radioactive sample measured by using different LSC protocols at different radioactive substances amount. The linearity formula obtained from each test is also presented.*

Sample (µL)	Don:Water=1:5 [^{3}H] DPM, Protocol 1	Water:Accep.=1:5 [^{14}C] DPM, Protocol 2	Don:Accep.=1:5 Protocol 3	
			[^{3}H] DPM	[^{14}C] DPM
0.2	38	185	27	203
0.5	72	538	50	505
1	134	1083	116	1050
2	386	2032	354	2114
4	833	4344	822	4289
6	1321	6161	1286	6468
8	1834	8313	1696	8004
10	2282	10283	2105	10169
12	2770	12764	2600	12506
14	3260	14487	2928	13882
20	4601	20358	4000	21475
30	7109	30498	6415	29540
40	9322	43841	8716	41675
50	11930	51141	10288	50028
Coefficient of regression (r^2)	0.9998	0.9981	0.9985	0.9987

The results show a good linearity in a wide range for both [^{3}H] and [14C] radiation measurement. For the radiation of [^{3}H], the DPM range from 20 to 12000 is proved to be linear. For the radiation of [^{14}C], the DPM range from 200 to 52000 is linear. Furthermore, the DPM values of [^{3}H] and [^{14}C] from a similar preparation counted by different protocols are quite comparable. No sample repetition is necessary due to the precision of the instrument. It can be concluded that the LSC measurement is quite reliable under the conditions used for the experiment.

6.2.3 Linearity test of Fluorescence Spectroscopy

6.2.3.1 Linearity test of rhodamine fluorescence

Rho standard solutions were prepared by dilution of the paclitaxel-containing donor liposome (DOTAP/DOPC/DOPE-Rho/PXL in the molar ratio 25/71/1/3) to a rhodamine concentration range from 0.83 x 10^{-2} to 26.7 x 10^{-2} μM. The rhodamine fluorescence intensity for different concentrations is presented in Table 6.4.

Table 6.4: *Fluorescence intensity measurement of rhodamine standard solutions in the concentration range from 0.83 x 10^{-2} to 26.7 x 10^{-2} μM. RSD values are calculated from three measurements at each concentration.*

[Rho] (x 10^{2} μM)	Fluorescence intensity (a.u.)
0.83	27.35 ± 0.44 %
1.67	56.53 ± 0.19 %
2.50	85.34 ± 0.21 %
3.33	113.09 ± 0.05 %
6.67	220.55 ± 0.50 %
13.33	425.47 ± 0.03 %
20.00	638.82 ± 0.31 %
26.67	843.88 ± 0.16 %

The fluorescence intensity presents a linear behaviour as a function of the rhodamine concentration between 0.83 x 10^{-2} and 26.7 x 10^{-2} μM (Intensity = 31.596c + 4.595, r^2=0.9999). The lysis of liposomes by addition of Triton X-100 does not give an increment of Rho intensity. Even though this phenomenon was not expected, it was not possible to further investigate the reason due to the limited batch produced by the company. Therefore, the Rho concentration in all eluents in the experiments is measured as it is, without further lysis by Triton X-100.

6.2.3.2 Linearity test of temoporfin fluorescence

The calibration of temoporfin (TP) fluorescence intensity curve and the effect of Triton X-100 were examined by using a specially prepared TP containing liposomes with extremely low TP content (DPPC / DPPG / TP in the weight ratio 90.0 / 10.0 / 0.2). The TP standard solutions were prepared by dilution of this liposome to a TP concentration range from 10 to 500 ng/ml. The influence of Triton X-100 in TP for the concentration of 10 to 200

ng/mL was also investigated. The fluorescence intensity for each concentration is presented in Table 6.5.

Table 6.5: *Fluorescence intensity of a low-TP-containing liposome suspension in the concentration range from 10 to 500 ng/ml and the effect of Triton X-100 to liposomes. Three measurements were performed for each concentration.*

C TP (ng/ml)	Fluorescence intensity (a.u.)	Fluorescence intensity (a.u.), after addition of 100 µL Triton X-100
10	61.13 ± 1.11 %	55.58 ± 0.25 %
25	95.10 ± 0.19 %	86.40 ± 0.30 %
50	151.36 ± 0.08 %	137.69 ± 0.27 %
100	273.93 ± 0.18 %	250.61 ± 0.06 %
150	381.40 ± 0.22 %	346.22 ± 0.08 %
200	482.56 ± 0.13 %	437.27 ± 0.11 %
250	564.37 ± 0.05 %	-
300	664.17 ± 0.11 %	-
350	749.86 ± 0.05 %	-
400	852.35 ± 0.02 %	-
500	989.34 ± 0.09 %	-

The fluorescence intensity shows a linear behaviour (Intensity = 1.9329c + 68.395, r^2=0.9945, n=11) within the TP concentration range from 10 to 500 ng/ml. The addition of 100 µL Triton X-100 to the standard solution for the concentration range from 10 to 200 ng/ml gives rise to decreased fluorescence intensity. The factor of 1.1 is used to correct the influence of Triton X-100 and the dilution effect of the samples.

6.3 Optimization of the ion-exchange micro-column

6.3.1 The optimal amount of saturation liposomes for ion-exchange gels

The selection of the optimal saturation liposome amount for both CM Sepharose FF and DEAE Sepharose CL-6B gel filled ion-exchange micro-columns was performed by pre-saturating the columns with 0 to 300 µL saturation liposomes. 10 µL of the mixture of donor acceptor liposomes were applied on these pre-saturated columns, and the recovery of donor and acceptor liposomes in the eluent after separation by the columns was evaluated. The optimal saturation liposome amount for the columns was selected as the columns with the best

separation efficiency. For CM Sepharose FF gel filled ion-exchange micro-columns, positively charged donor liposomes (DOTAP/DOPC/Chol/[^{3}H]COE in the molar ratio of 25/72/3) and acceptor liposomes (POPC/Chol/[^{14}C] CO in the molar ratio of 97/3) were used for the experiment. For DEAE Sepharose CL-6B gel filled ion-exchange micro-columns, negatively charged donor liposomes (DCP/DOPC/Chol/[^{3}H]COE in the molar ratio of 2/7/1) and acceptor liposomes (POPC/Chol/[^{14}C] CO in the molar ratio of 8/2) were used for the experiment. The donor and acceptor liposomes were of the same lipid concentration (10 mM), and acceptor liposomes were in 5 times excess. The recovery of donor and acceptor liposomes in the eluent separated by the columns pre-saturated with different saturation liposomes amount is presented in Table 6.6 and Table 6.7 respectively.

Table 6.6: *Percentage recovery of donor (DOTAP/DOPC/Chol/[^{3}H]COE=25/72/3, molar ratio) and acceptor (POPC/Chol/[^{14}C] CO=97/3, molar ratio) liposomes in the eluent after separation through CM Sepharose FF gel filled ion-exchange micro-columns at different amounts of saturation liposomes. RSD values are calculated from three repetitions.*

Saturation liposomes amount (μL)	Donor recovery (%) by CM Sepharose FF gel	Acceptor recovery (%) by CM Sepharose FF gel
0	0.02 ± 40.00 %	86.33 ± 1.05 %
10	0.18 ± 33.33 %	87.64 ± 0.87 %
20	0.27 ± 33.33 %	89.69 ± 1.07 %
30	0.46 ± 17.39 %	89.29 ± 0.86 %
40	0.50 ± 18.00 %	91.25 ± 1.03 %
50	0.53 ± 18.87 %	90.41 ± 1.28 %
100	0.51 ± 13.73 %	88.68 ± 2.86 %
300	1.15 ± 6.96 %	88.33 ± 2.27 %

The results show that the columns without saturation give the lowest donor and acceptor recoveries. It can be explained through that a certain amount of donor and acceptor liposomes are adsorbed inside some gaps of the freshly packed ion-exchange gels or that the liposomes are destroyed by hydrophobic binding places on the gel particles. Therefore it is necessary to saturate the gel to reduce the non-specific adsorption of the gel and to improve the recovery of acceptor liposomes before performing the experiments.

The increase of saturation liposome amounts from 10 to 50 μL is followed by a increase of acceptor recovery from 87 % to 91 %. A further increase of saturation liposome amount to

100 or 300 µL gives a slightly decreased acceptor recovery of around 88 %. An increasing trend of the donor recovery is observed during the augmentation of saturation liposome amount. The donor recovery increased first from 0.02 to 0.46 % when the saturation liposomes amount reaches 30 µL, and then keeps relatively stable at around 0.50 % when the saturation liposome amount varied from 40 to 100 µL. A sharp increment of donor recovery up to 1.15 % is obtained when the saturation liposome amount is increased to 300 µL. This can be explained through that the exceeded amount of saturation liposomes occupies some adsorption positions of donor liposomes, maybe by masking the charges on the gel particles. The highest acceptor recovery and the most stable donor recovery are obtained at the 40 µL or 50 µL saturation liposome amount.

Table 6.7: *Recovery of donor (DCP/DOPC/Chol/[^{3}H]COE=2/7/1, molar ratio) and acceptor (POPC/Chol/[^{14}C] CO=8/2, molar ratio) liposomes in the eluent after separation through DEAE Sepharose CL-6B gel filled ion-exchange micro-columns at different amount of saturation liposomes.* R*SD values are calculated from three repetitions.*

Satu. liposome (µL)	Don. recovery (%) by DEAE Sepharose CL-6B gel	Acc. recovery (%) by DEAE Sepharose CL-6B gel
0	0.25 ± 24.00 %	78.31 ± 3.09 %
10	0.37 ± 21.62 %	82.14 ± 1.25 %
20	0.79 ± 13.92 %	83.58 ± 0.31 %
30	0.96 ± 15.62 %	84.02 ± 1.14 %
40	1.01 ± 3.96 %	84.95 ± 1.61 %
50	1.03 ± 6.79 %	86.63 ± 0.88 %
100	1.10 ± 9.09 %	84.60 ± 1.23 %
300	2.15 ± 10.69 %	84.75 ± 1.75 %

A similar phenomenon is observed in DEAE Sepharose CL-6B gel filled ion-exchange micro-columns. The columns without pre-saturation give the lowest acceptor recovery. The acceptor recovery first increases from 82 to 86 %, and then goes back to 84 % when the saturation liposome amount reaches 100 and 300 µl. The highest acceptor recovery is achieved at 50 µl of the saturation liposome amount. The recovery of donor liposomes keeps increasing from 0.37 to 0.96 % when the saturation liposomes amount increases from 10 to 30 µl, and keeps constant at around 1 % when the amount of saturation liposomes is in the range from 40 to

100 µl. An excess of 300 µl saturation liposome amount shows an apparent increment in donor recovery.

Compared to the CM Sepharose FF gel filled ion-exchange micro-columns, the DEAE Sepharose CL-6B gel filled ion-exchange micro-columns have a generally lower acceptor recovery. The tendency of donor and acceptor recovery change with the increase of saturation liposome amount is similar in both cases. An overburdened amount of saturation liposomes might give rise to a decline in acceptor liposome recovery.

The saturation liposome amount of 50 µl is finally selected for reducing non-specific adsorption. All the columns used in the experiments are pre-saturated by 50 µl of saturation liposomes before the performance of the experiments.

6.3.2 Maximal liposome application amount on the columns

The liposome application amount for the cholesterol and cholesteryl-oleoyl-ether transfer kinetics study is within the recommended liposome application amount. Therefore, it is not necessary to look for the maximal application amount in cholesterol and cholesteryl-oleoyl-ether transfer study. The maximal liposome loading amount is only needed in paclitaxel transfer kinetics study because of the restricted detection limit of HPLC. The experiments were therefore designed for the paclitaxel transfer kinetics study.

The selection of the maximal liposome application amount on the columns was performed by applying from 10 to 60 µL liposome mixtures on the CM Sepharose FF gel filled columns. Donor blank liposomes used in the incubation were DOTAP/DOPC/DOPE-Rho (molar ratio 25/74/1, 10 mM lipid concentration). The radio-labelled acceptor liposomes were composed of POPC/[^{14}C] CO with the lipid concentration of 10 mM or 50 mM. In order to investigate if there is an influence of incubation time to the donor blank and acceptor liposomes, the donor and acceptor liposomes separated immediately after incubation, and separated 30 min after incubation were examined. The recovery of donor liposomes was measured as the recovery of rhodamine by Fluorescence Spectroscopy, and the recovery of acceptor liposomes was measured as the recovery of [^{14}C] CO by LSC. Table 6.8 shows the recovery of donor liposomes from five incubation groups I) Donor:water = 1:1 (v/v); II) Donor (10 mM):Acceptor (10 mM) = 1:1 (v/v) separated by the ion-exchange micro-columns immediately after mixing; III) Donor (10 mM):Acceptor (10 mM) = 1:1 (v/v) separated after 30 minutes incubation time; IV) Donor (10 mM):Acceptor (50 mM) = 1:1 (v/v) separated immediately after mixing; V) Donor (10 mM):Acceptor (50 mM) = 1:1 (v/v) separated after 30 minutes incubation time.

Table 6.8: *Recovery of donor blank liposomes calculated by Rho fluorescence measurement from the donor lipids in eluent at different liposome application amounts. The data are obtained from the incubation of donor blank liposomes (DOTAP/DOPC/DOPE-Rho = 25/74/1, molar ratio, 10 mM lipid concentraiton) and radio-labelled acceptor liposomes (POPC/[^{14}C] CO, 10 or 50 mM lipid concentrations). The results are presented as the average ± RSD, and calculated from six repetitions.*

Sample (µL)	Don + Water	Don + Accep (10 mM), 0 min	Don+ Accep (10 mM), 30 min	Don + Accep (50 mM), 0 min	Don + Accep (50 mM), 30 min
10	0.64 ± 29.68 %	2.05 ± 2.44 %	1.98 ± 32.82 %	2.83 ± 26.85 %	2.81 ± 8.89 %
20	1.02 ± 14.71 %	1.88 ± 30.31 %	1.41 ± 32.62 %	3.69 ± 2.11 %	3.75 ± 18.4 %
30	1.53 ± 49.01 %	1.24 ± 52.41 %	1.47 ± 18.36 %	2.72 ± 24.26 %	3.28 ± 31.09 %
40	1.04 ± 9.61 %	2.15 ± 34.88 %	2.53 ± 13.44 %	3.76 ± 17.71 %	3.23 ± 22.29 %
50	1.14 ± 47.36 %	1.64 ± 52.43 %	2.97 ± 11.45 %	3.98 ± 7.03 %	4.42 ± 6.78 %
60	1.16 ± 10.34 %	1.28 ± 49.84 %	1.89 ± 12.69 %	3.82 ± 31.77%	4.33 ± 15.70 %

According to the data in Table 6.8, an schematic graph of donor liposome recovery against liposome application amounts is illustrated in Figure 6.2.

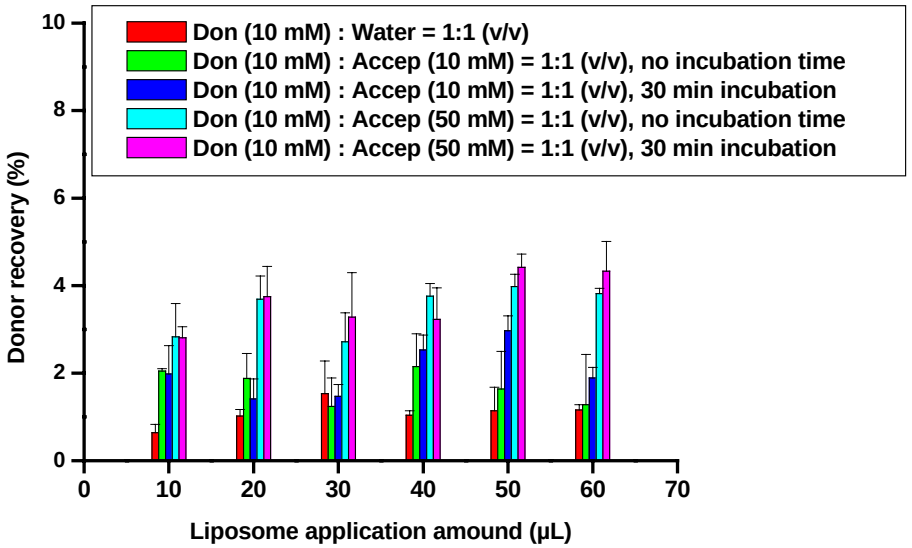

Figure 6.2: *Schematic graph of donor liposome recovery against the applied liposome amount according to Table 6.8. Error bars are calculated from six repetitions.*

As shown in Figure 6.2, the lowest donor recovery is obtained from the control experiment, where donor is incubated with water, at the absence of acceptor liposomes. The presence of acceptor liposomes increases the donor recovery in the eluent. Additionally, the higher donor

recovery is obtained when donor is incubated with acceptor liposomes with a higher acceptor lipid concentration. What is important is that the increase of liposome application amount does not give rise to a serious increase in donor liposome recovery in all the incubation groups. Only in the incubation group of donor with 50 mM acceptor liposomes, a slightly increase of donor liposome recovery in the eluent is observed. The 30 min incubation time does not influence the recovery of donor liposomes. Under the experimental conditions, the donor recovery is overall less than 5 %, which proves a promising retention ability of CM Sepharose FF gel filled ion-exchange micro-columns for the positively charged donor liposomes. The acceptor liposome recovery measured by LSC from each incubation group at different liposome application amounts is gathered in Table 6.9.

Table 6.9: *The recovery of acceptor liposomes calculated by [^{14}C]CO radioactivity counting in eluent at different liposome application amounts. The data are obtained from the incubation of donor blank liposomes (DOTAP/DOPC/DOPE-Rho = 25/74/1, molar ratio, 10 mM lipid concentration) and radio-labelled acceptor liposomes (POPC/[^{14}C] CO, 10 or 50 mM lipid concentrations). The results are presented as the average ± RSD, and calculated from six repetitions.*

Sample (μL)	Don + Accep (10 mM), 0 min	Don + Accep (10 mM), 30 min	Don + Accep (50 mM), 0 min	Don + Accep (50 mM), 30 min
10	84.92 ± 5.38 %	83.73 ± 7.42 %	85.52 ± 6.19 %	85.41 ± 1.04 %
20	93.65 ± 1.77 %	91.30 ± 4.08 %	84.88 ± 4.31 %	85.84 ± 1.43 %
30	84.88 ± 1.90 %	87.01 ± 0.15 %	88.79 ± 6.56 %	89.72 ± 3.97 %
40	89.88 ± 0.45 %	90.91 ± 5.10 %	87.91 ± 5.79 %	85.64 ± 1.70 %
50	90.6 ± 0.38 %	92.81 ± 4.85 %	90.29 ± 3.34 %	88.06 ± 1.79 %
60	90.38 ± 3.66 %	89.29 ± 5.86 %	84.86 ± 6.08 %	87.18 ± 2.37 %

The data gathered in Table 6.9 are represented in the schematic graph of acceptor recovery against liposome application amounts of Figure 6.3.

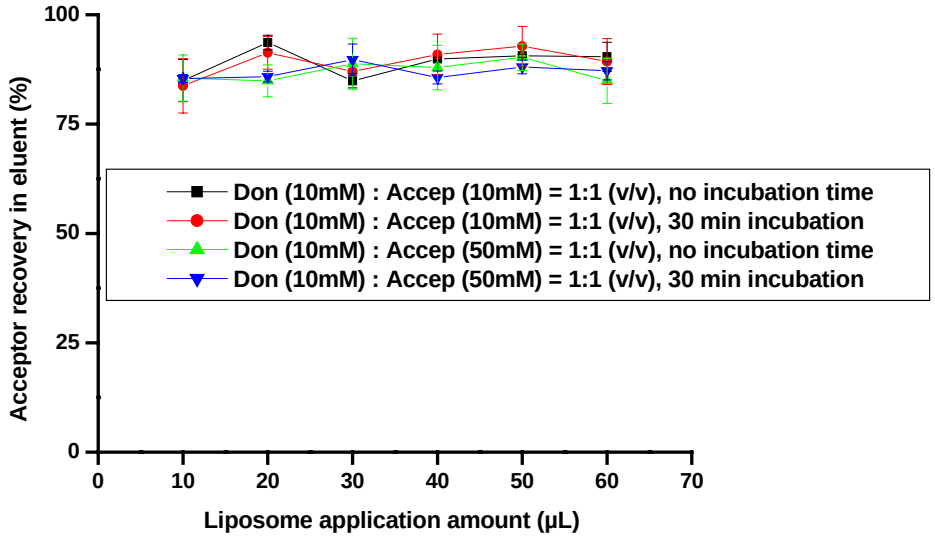

Figure 6.3: *Overlay of acceptor liposome recovery in eluent calculated by [^{14}C]CO radioactivity against the applied liposome amount according to Table 6.9. Error bars are calculated from six repetitions.*

The recovery of acceptor liposomes as shown in Figure 6.3 from all the incubation groups is in the range from 83 to 93 %. The difference in acceptor lipids concentration, the increase in sample application amount and the incubation time do not influence the acceptor liposome recovery in the eluent. The ion-exchange micro-columns show an effective ability to separate acceptor liposomes from donor liposomes with an acceptor liposome recovery in the range from 83 to 93 %, and a donor blank liposome recovery in the eluent of less than 5 %.

The results show that under these experimental conditions, a successful separation between donor blank and acceptor liposomes can still be obtained even when the liposome application amount reaches up to 60 µL. Under the consideration of both donor and acceptor recovery in the eluent, the sample application amount of 50 µL is chosen for studying paclitaxel transfer between liposomal membranes, which is sufficient for being detected by HPLC.

6.3.3 The selection of donor acceptor incubation ratio for CM Sepharose FF gel filled micro-columns

The selection of the donor acceptor incubation ratio was performed by incubation of three types of donor blank liposomal formulations with different concentrations of acceptor liposomes. The donor blank liposomes were DOTAP/DOPC/DOPE-Rho (5/94/1, molar ratio), DOTAP/DOPC/DOPE-Rho (25/74/1, molar ratio) and DOTAP/DOPC/DOPE-Rho/DOPE-PEG (25/69/1/5, molar ratio). The abbreviations Don. (5 % DOTAP), Don. (25 % DOTAP)

and Don. (25 % DOTAP + 5 % PEG) are used to simplify these donor blank liposomes. All the donor liposomes had a lipids concentration of 10 mM. The acceptor POPC/[^{14}C] CO liposomes had the lipids concentration of 50 mM. Acceptor liposomes at lower lipids concentrations were obtained by dilution from acceptor liposomes of 50 mM. The recovery of donor liposomes was measured as the recovery of rhodamine (Rho) by fluorescence spectroscopy, and the recovery of acceptor liposomes was measured as the recovery of [^{14}C] CO by LSC. Table 6.10 shows the recovery of donor liposomes from each incubation group after 30 min incubation time at different acceptor lipids concentrations.

Table 6.10: *Donor liposome recovery calculated by Rho fluorescence measurement from donor lipids in eluent with the increase of acceptor lipids concentration. The data are obtained from the incubation of three donor blank liposomal formulations: Don. (5 % DOTAP), Don. (25 % DOTAP) and Don. (25 % DOTAP + 5 % PEG) and radio-labelled acceptor liposomes (POPC/[^{14}C] CO). The results are presented as the average ± RSD, and calculated from six repetitions.*

[Acceptor lipids] (mM)	Don (5% DOTAP)	Don (25% DOTAP)	Don (25% DOTAP + 5% PEG)
0	0.81 ± 11.11 %	0.87 ± 17.24 %	0.91 ± 14.28 %
10	2.85 ± 5.26 %	2.29 ± 20.08 %	2.46 ± 25.37 %
20	5.37 ± 28.30 %	2.34 ± 20.07 %	4.49 ± 30.06 %
30	7.60 ± 19.34 %	3.35 ± 22.68 %	6.32 ± 16.93 %
40	9.94 ± 16.09 %	3.74 ± 10.16 %	8.56 ± 11.56 %
50	12.79 ± 11.25 %	3.18 ± 19.18 %	10.22 ± 8.90 %

Donor liposome recovery against acceptor lipids concentration of Table 6.10 is represented in Figure 6.4.

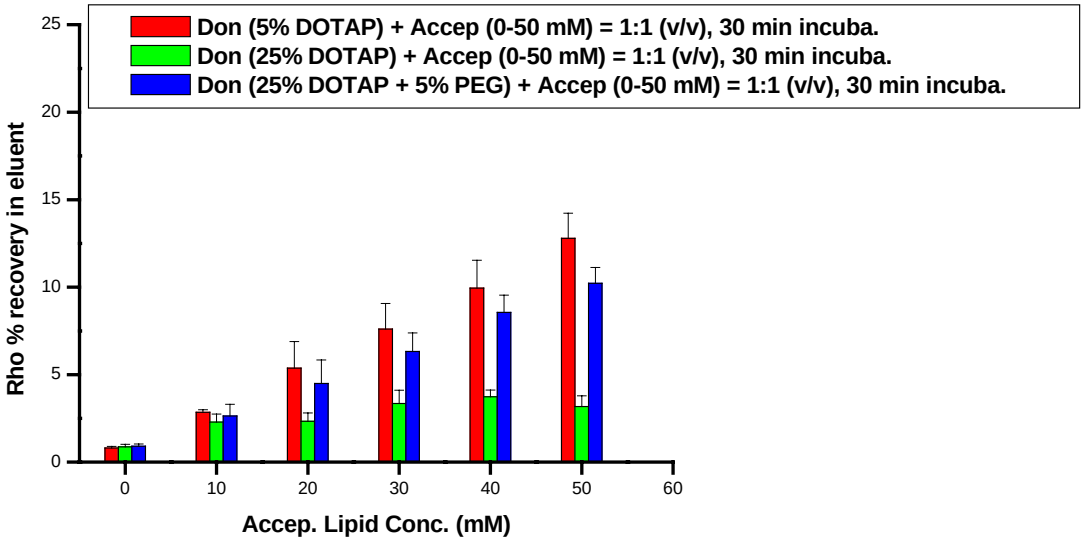

Figure 6.4: *Donor recovery after 30 min incubation time calculated by rhodamine fluorescence measurement from donor lipids in eluent against acceptor lipid concentration referring to Table 6.10. Error bars are calculated from six repetitions.*

As seen in Figure 6.4, in the case of the incubation of donor (25 % DOTAP) and acceptor, the recovery of donor liposomes is relatively constant with the increase of the acceptor lipids concentration. However, a noticeable increase of donor liposome recovery in the eluent with the increasing of acceptor lipids concentration is detected in the cases of incubation of donor (5 % DOTAP) or donor (25 % DOTAP + 5 % PEG) with acceptor liposomes. It is worth to mention that, the zeta-potential of donor (5 % DOTAP) and donor (25 % DOTAP + 5 % PEG) is +26.6 and +32.5 mV, respectively, which are only half of the value of the zeta-potential of donor (25 % DOTAP) liposomes of +65.1 mV. Positively charged donor liposomes with zeta-potential not more than +32.5 mV seem to have a weaker interaction with the ion-exchange gel. The increase of neutral acceptor lipid concentration might further prevent the donor-gel interaction by surrounding the positively charged donor liposomes and keep them away from contact with the negative ion-exchange gel. This decreased donor-gel interaction can be expressed as an increase of donor recovery in the ion-exchange micro-column experiment. Therefore, the insufficient zeta-potential of donor (5 % DOTAP) and donor (25 % DOTAP + 5 % PEG) liposomes is responsible for the increasing trend of donor recovery with the increase in acceptor lipid concentration.

The acceptor liposome recovery measured by LSC from each incubation group at different acceptor lipid concentrations is gathered in Table 6.11, and the schematic graph of acceptor recovery against acceptor lipids concentrations is illustrated in Figure 6.5.

Table 6.11: *Acceptor liposome recovery calculated by [14C]CO radioactivity counting in eluent with the increase of acceptor lipids concentration. The data are obtained from the incubation of three donor blank liposomal formulations: Don. (5 % DOTAP), Don. (25 % DOTAP) and Don. (25 % DOTAP+5 % PEG) and radio-labelled acceptor liposomes (POPC/[^{14}C] CO). The results are presented as the average ± RSD, and are calculated from six repetitions.*

[Acceptor lipids] (mM)	Acceptor recovery (%) from Don (5% DOTAP)	Acceptor recovery (%) from Don (25% DOTAP)	Acceptor recovery (%) from Don (25% DOTAP + 5% PEG)
10	92.40 ± 3.30	86.80 ± 3.67	89.67 ± 2.78
20	92.41 ± 1.91	90.78 ± 3.58	85.49 ± 2.30
30	89.02 ± 3.38	90.67 ± 3.53	86.29 ± 4.75
40	90.62 ± 2.18	93.38 ± 2.09	91.04 ± 3.87
50	93.48 ± 2.14	91.68 ± 3.82	88.67 ± 2.88

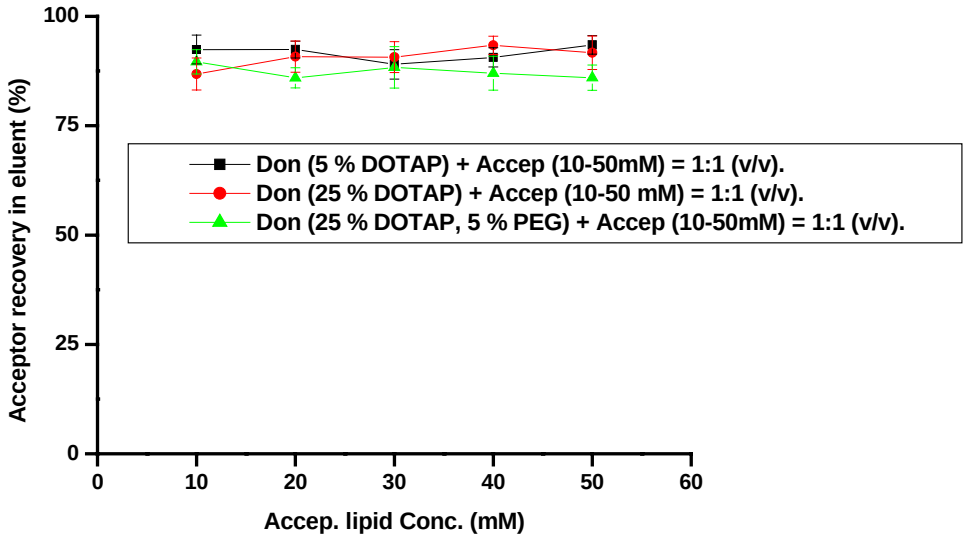

Figure 6.5: *Overlay of acceptor liposome recovery in eluent after 30 min incubation time calculated by [14C]CO radioactivity against acceptor lipids concentration, according to Table 6.11. Error bars are calculated from six repetitions.*

The recovery of acceptor liposomes in the eluent from all the incubation groups as shown in Figure 6.5 is constant within the range from 85 to 93 %. The acceptor liposome recovery from acceptor blank experiments, i.e., acceptor incubated with water, in the absence of donor

liposomes, is within the range from 90 to 92 % (data are now shown). All the results prove that the change in acceptor lipids concentration does not influence the recovery of acceptor liposomes. The acceptor liposomes can be recovered quite effectively by the ion-exchange micro-columns.

The donor and acceptor recovery results obtained in this experimental design show that, one of the pre-conditions for a successful donor acceptor separation by the ion-exchange micro-columns is the sufficient zeta-potential of donor liposomes. The zeta-potential not more than +32.5 mV could not ensure a successful donor acceptor separation. The results from donor (25 % DOTAP) and acceptor liposome incubation experiment show that, an approximately 90 % acceptor recovery and around 3 % donor recovery can be obtained regardless of the increase of acceptor lipids increase. As it can be concluded from the donor (25 % DOTAP) and acceptor incubation experiment, if there is good enough donor-gel interaction, the donor liposome can be separated effectively from the acceptor liposomes even when the acceptor lipids concentration increases up to 5 times in excess to donor lipids concentration. Therefore, the DOTAP concentration of 25 % is selected for the paclitaxel-containing liposomal formulations DOTAP/DOPC/DOPE-Rho/PXL (molar ratio 25/71/1/3) and DOTAP/DOPC/DOPE-Rho/DOPE-PEG/PXL (molar ratio 25/66/1/5/3) to ensure a sufficient zeta-potential of the donor liposomes. However, in the case of long circulating paclitaxel-containing donor liposomes, the zeta-potential turns to be only +24.8 mV due to the incorporation of PEG. This can be a reason to explain the low separation efficiency of donor (PXL-PEG) and acceptor liposomes of the experiments performed in Section 6.4. The acceptor lipid concentration is prepared at 50 mM, and incubated with 10 mM donor liposomes in the same volume to ensure a 5-times excess of acceptor liposomes to donor liposomes.

Summary

All the liposomes prepared for the experiments meet the quality control. For the liposomes used in the ion-exchange micro-column experiments, their zeta-potential plays an essential role in the success of the experiment. The ratio of DOTAP in lipid composition determines the zeta-potential of the liposomes. A DOTAP concentration of 5% gives rise to a zeta-potential of only +25 mV, which is not sufficient for the ion-exchange transfer experiment. The addition of PEG results in a decrement in zeta-potential. The addition of PXL does not influence the zeta-potential of the liposomes and the optimal positive (or negative) zeta-potential is selected by adjusting the concentration of DOTAP (or DCP).

The fusion of the liposomes in the ion-exchange micro-column experiments can be excluded Based on the TEM characterization in the PXL transfer study, the PCS control of the donor and acceptor liposomes before and after the separation by ion-exchange micro-columns and the no-fusion assumption according to previous results [Haran N. and Shporer M., (1977)]. Therefore, the ion-exchange micro-column model proved to be a reliable *in vitro* method for studying the transfer of drugs between liposomal membranes.

The optimization of the ion-exchange micro-column is considered as the pre-experiment before the performance of the transfer of different drugs between liposomal membranes. In this pre-experiment, the most important parameters, which influence the reliability and sensitivity of the drug transfer model, are selected. The study of the transfer kinetics of paclitaxel is regarded as the key task in the study. The transfer kinetics of all the other compounds, i.e. cholesterol and cholesteryl-oleoyl-ether, is compared with the transfer kinetics of paclitaxel. Due to the limitation in the paclitaxel measurement by HPLC, the optimal experimental conditions should be specially selected to ensure a reliable quantification. The parameters used in the cholesterol and choelsteryl-oleoyl-ethyl transfer studies are adjusted to be in accordance with the parameters used in the paclitaxel transfer study. The final experimental parameters for the drug transfer are selected as follows:

i) 50 µL of saturation liposomes for the pre-treatment of the ion-exchange micro-columns.

ii) the donor acceptor incubation ratio of 1 to 5.

iii) 50 µL (only in PXL transfer) of sample, which are applied on the columns.

In the case of paclitaxel transfer study, 10 mM donor liposomes and 50 mM acceptor liposomes incubated are used in an equal volume. In the case of cholesterol and cholesteryl-oleoyl-ether transfer study, 10 mM donor liposomes and 10 mM acceptor liposomes are incubated in 1 to 5 volume ratio in order to reach the final donor acceptor incubation of 1 to 5. Due to the high sensitivity of LSC for measuring the transfer of cholesterol and cholesteryl-oleoyl-ether, only 10 µL of sample are enough to get a good resolution of donor and acceptor. The amount of 10 µL instead of 50 µL is then used in the transfer of cholesteorl and cholesteryl-oleoyl-ether experiments. Under these selected conditions, the transfer kinetics of the three compounds, paclitaxel, cholesterol and cholesteryl-oleoyl-ether, is investigated in the following three sections.

6.4 Paclitaxel transfer between liposomal membranes

6.4.1 Linear relation of paclitaxel transfer

The experiment was studied by incubating donor with acceptor of different lipid concentrations. Two paclitaxel-containing donor liposomes: DOTAP/DOPC/DOPE-Rho/PXL in the molar ratio of 25/71/1/3, named as Donor (PXL) and DOTAP/DOPC/DOPE-Rho/DOPE-PEG/PXL in the molar ratio of 25/66/1/5/3, named as Donor (PXL-PEG) were used in the experiment. Both non-radioactive POPC liposomes and Cholesteryl [1-^{14}C] oleate labelled radioactive POPC/ [^{14}C] CO liposomes for a lipid concentration of 50 mM were used as acceptor liposomes. Acceptor liposomes at lower lipid concentrations were obtained by dilution from acceptor liposomes of 50 mM. Three incubation groups were designed as I) donor (10 mM) : water = 1 : 1 (v/v), II) acceptor (10, 20, 30, 40 and 50 mM) : water = 1 : 1 (v/v) and III) Donor (10 mM) : Acceptor (10, 20, 30, 40 and 50 mM) = 1 : 1 (v/v). After 30 min incubation, the acceptor liposomes were separated from the donor liposomes. The recovery of donor liposomes was measured by fluorescence spectroscopy, the data were calculated from six repetitions (three times incubation of donor with non-radioactive POPC acceptor and three times incubation of donor with radioactive POPC [^{14}C]CO acceptor). The recovery of radioactive acceptor liposomes was measured as the recovery of [^{14}C] CO by LSC, from the three donor with non-radioactive POPC acceptor incubations. The transfer amount of paclitaxel was measured by HPLC/UV, from the three incubations of donor and non-radioactive acceptor liposomes. The results of donor recovery from donor (PXL) and donor (PEX-PEG) liposomal formulations with the increase of acceptor lipid concentrations are gathered in Table 6.12.

Table 6.12: *Recovery of donor (PXL) and donor (PXL-PEG) liposomes calculated by rhodamine fluorescence from donor lipids in eluent against the increase of acceptor lipids concentrations after 30 min incubation. The results are presented as the average ± RSD, and calculated from six repetitions.*

[Acceptor lipid] (mM)	Donor recovery (%) Don (PXL)	Donor recovery (%) Don (PXL-PEG)
0	3.34 ± 12.87 %	14.86 ± 19.98 %
10	6.49 ± 28.04 %	17.52 ± 18.09 %
20	5.24 ± 39.42 %	17.02 ± 11.16 %
30	5.79 ± 14.85 %	15.48 ± 31.45 %
40	5.54 ± 33.21 %	14.17 ± 12.42 %
50	9.69 ± 46.02 %	19.03 ± 29.26 %

The schematic graph of donor liposome recovery measured as Rho recovery from two types of donor liposomes against acceptor lipids concentration is illustrated in Figure 6.6.

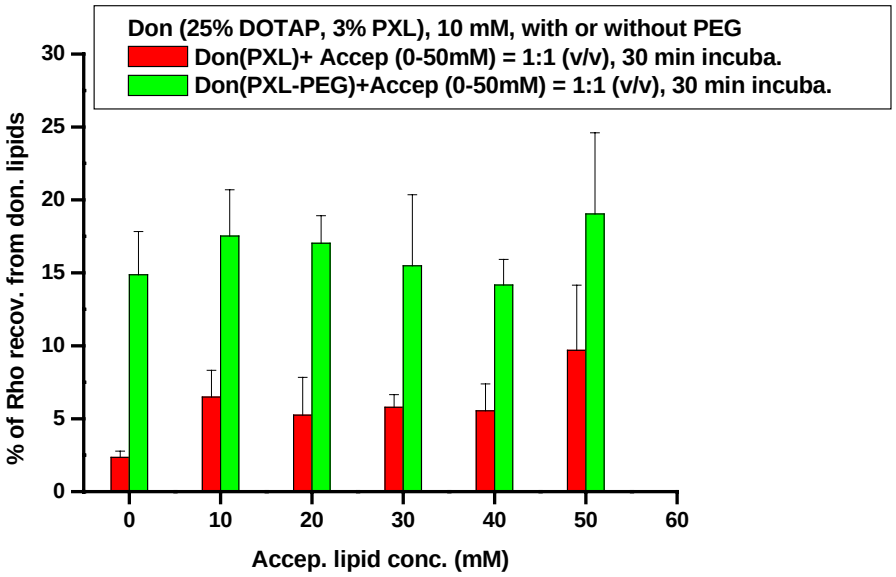

Figure 6.6: *Recovery of donor (PXL) and donor (PXL-PEG) calculated by rhodamine fluorescence measurement from donor lipids in eluent against acceptor lipids concentration after 30 min incubation, according to Table 6.12. Error bars are calculated from six repetitions.*

As shown in Figure 6.6, around 3 % donor is recovered in the eluent at the absence of acceptor liposomes. The donor recovery keeps stable at around 5 % when acceptor lipid concentration varied from 10 to 40 mM. A slightly increment of donor (PXL) recovery up to approximately 9 % in the eluent is observed when the acceptor lipid concentration reaches 50 mM. The recovery of donor (PXL) liposomes (lipid composition: DOTAP/DOPC/DOPE-Rho/PXL in a molar ratio of 25/71/1/3) is generally higher than the recovery of the non-paclitaxel-containing donor (25 % DOTAP) liposomes (lipid composition: DOTAP/DOPC/DOPE-Rho in molar ratio of 25/74/1) in a similar experimental design in Section 6.3.3. Even though there is only 3 mV difference in the zeta-potential of donor (PXL) and donor (25 % DOTAP) liposomes (+ 61.4 and + 65.1 mV, respectively), a higher percentage of donor (PXL) liposomes are recovered in the eluent. When the acceptor lipid concentration increases to 50 mM, the donor (PXL) recovered in the eluent reaches nearly 10 %. This indicates that, the maximal acceptor lipid concentration should not exceed 50 mM, in order to guarantee an effective donor acceptor separation by the ion-exchange micro-columns.

A similar tendency is found in the incubation of donor (PXL-PEG) with acceptor liposomes. The recovery of donor (PXL-PEG) liposomes in the eluent is generally much higher than the recovery of donor (PXL) liposomes. When donor (PXL-PEG) is only incubated with water, more than 14 % of the donor liposomes are already found in the eluent. The recovery of donor (PXL-PEG) varies within the range from 14 % to 17 % when the acceptor lipid concentration increases from 10 to 40 mM. When the acceptor lipid concentration reaches 50 mM, the recovery of donor (PXL-PEG) increases up to 19 %. The data indicate an inadequate interaction of donor liposomes with the gel. In a similar experimental design in Section 6.3.3, where the non-paclitaxel-containing donor (25 % DOTAP + 5 % PEG) liposomes (lipid composition: DOTAP/DOPC/DOPE-Rho/DOPE-PEG in a molar ratio of 25/69/1/5) was incubated with different concentration of acceptor liposomes, an increase trend of donor (25 % DOTAP + 5 % PEG) recovery with the increase of acceptor lipid concentration is observed. However, in the case of donor (PXL-PEG) incubation, the increasing tendency is not so much pronounced. It is worth to mention that, the zeta-potential of donor (PXL-PEG) and donor (25 % DOTAP + 5 % PEG) is + 24.8 and + 32.5 mV, respectively. The dramatic decrease in zeta-potential of the donor (PXL-PEG) might be responsible for the difference in the donor recovery from these two incubations. The zeta-potential of donor (PXL-PEG) liposomes could not ensure a sufficient interaction of donor liposomes with the ion-exchange gel even at the absence of neutral acceptor vesicles. Therefore, the increase in acceptor lipid concentration does not give rise to a further obvious inhibition of the interaction between donor and the ion-exchange gel, until the acceptor lipid concentration reaches 50 mM. With a higher acceptor lipid concentration, the donor liposomes might be covered by the concentrated acceptor liposomes, and the interaction of donor with ion-exchange gel is further deteriorated. As a result, there is a noticeable increase of donor (PXL-PEG) recovery at the acceptor lipid concentration of 50 mM.

For acceptor recovery measurement, only the recovery of [^{14}C] CO labelled radioactive acceptor liposomes was measured by LSC. The recovery of non-radioactive labelled acceptor POPC liposomes was not measured and was regarded to be comparable with the recovery of radioactive acceptor liposomes. The acceptor POPC [^{14}C] CO liposome recovery from the incubation of donor (PXL) or donor (PXL-PEG) with acceptor liposomes at different acceptor lipid concentrations is gathered in Table 6.13.

Table 6.13: *Radio-labelled acceptor POPC [^{14}C] CO liposome recovery calculated by [^{14}C]CO radioactivity counting in eluent with the increase of acceptor lipids concentration. The results are presented as average ± RSD, and obtained from three repetitions.*

[Acc. lipid] (mM)	Acc. recovery (%) Don. (PXL) : Acc. = 1 :1 (v/v)	Acc. recovery (%) Don. (PXL-PEG) : Acc. = 1:1 (v/v)
10	90.01 ± 5.73 %	87.86 ± 4.74 %
20	88.75 ± 4.22 %	86.94 ± 5.55 %
30	87.96 ± 4.99 %	90.21 ± 5.80 %
40	92.34 ± 6.38 %	89.01 ± 4.42 %
50	88.47 ± 4.78 %	91.36 ± 5.66 %

According to the data in Table 6.13, the schematic graph of acceptor recovery from the incubation of donor (PXL) or donor (PXL-PEG) with acceptor liposomes against the acceptor lipid concentrations is illustrated in Figure 6.7.

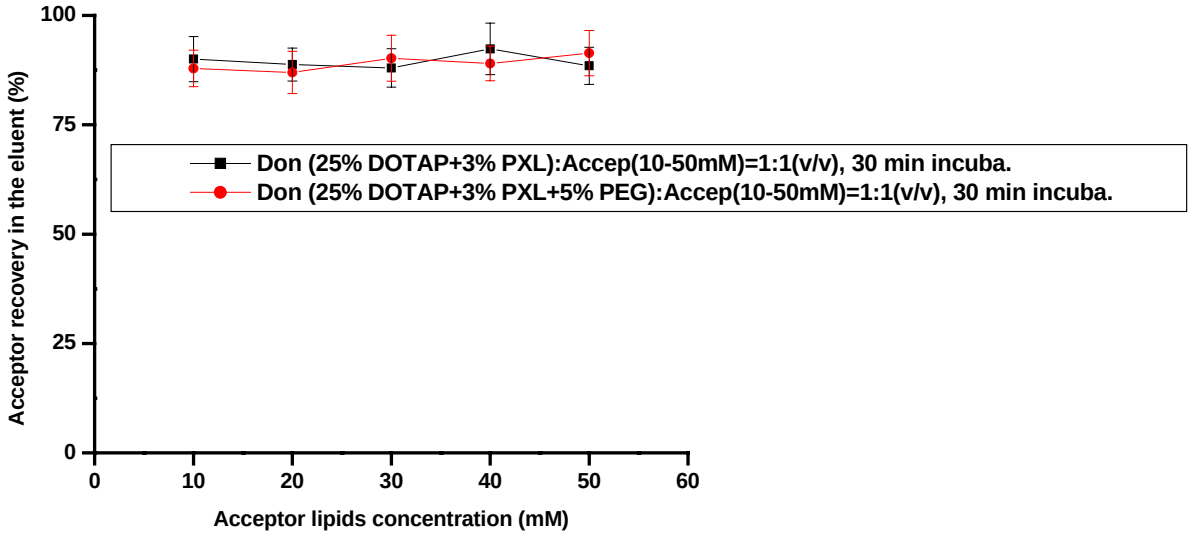

Figure 6.7: *Overlay of radio-labelled acceptor POPC [^{14}C] CO liposome recovery in eluent calculated by [14C]CO radioactivity against acceptor lipids concentration according to Table 6.13. Error bars are calculated from three repetitions.*

The recovery of acceptor liposomes in the eluent from both incubation groups as shown in Figure 6.7 is constant within the range from 86 to 92 %. When the acceptor liposomes with different lipid concentrations are incubated with water, a similar acceptor recovery in the range from 90 to 92 % was observed (data are not shown). The incubation of acceptor with or

without donor liposomes does not influence the recovery of acceptor liposomes. The type of donor liposomes with different zeta-potentials has no significant influence on the recovery of acceptor liposomes. In all the incubation groups, the acceptor liposomes can be recovered quite effectively by the ion-exchange micro-columns.

The lipid used for donor liposomes is DOPC, and for acceptor liposomes is POPC. Both are unsaturated phospholipids. These two lipids differentiated in one of their carbon chains. Under the experimental conditions, both lipids are in a fluid state. As reported in the literature, the water solubility of paclitaxel is found to be 0.50 ± 0.05 µM [Wenk M.R. et al., (1996)]. That is to say that there is always the equilibrium of 0.5 µM paclitaxel dissolved in water. Under this condition, and together with the assumption that paclitaxel is equally distributed between donor and acceptor liposomes, the transfer of paclitaxel from PXL-containing donor liposomes should be proportionally linear to the ratio of [Acceptor]/([Donor] + [Acceptor]). Based on these assumptions, the experiment was designed to verify this linear relation.

The recovery of non-radioactive acceptor liposomes is considered to be similar as the recovery of radioactive acceptor liposomes in a parallel experimental design. According to the values of radioactive acceptor liposomes recovery obtained in Figure 6.7, the value of 89 % is fixed as a constant recovery for the non-radioactive acceptor liposomes in the donor acceptor incubation experiment. Therefore, the obtained experimental paclitaxel transfer values should be normalized according to the acceptor recovery to 100 % (see Appendix 1 for a detailed explanation). Table 6.14 presents the theoretical values of paclitaxel transferred from donor to acceptor, the experimental PXL transfer data measured by HPLC in the incubation of donor (PXL) or donor (PXL-PEG) liposomes with acceptor (10 - 50 mM) liposomes.

According to the data in Table 6.14, the linear fits of theoretical values and the experimental values after normalization of the PXL transfer from donor (PXL) into acceptor POPC liposomes against the ratio of acceptor concentration to the sum of donor and acceptor concentration is illustrated in Figure 6.8.

Table 6.14: *Theoretical values, $[PXL]_{Th}$, experimental values of PXL transfer from donor (PXL) liposomes into acceptor liposomes after normalization, $[PXL]_{Acc}$ norm, and experimental values of PXL transfer from donor (PXL-PEG) liposomes into acceptor liposomes after normalization, $[PXL\text{-}PEG]_{Acc}$ norm. Donor (PXL) formulation is DOTAP/DOPC/DOPE-Rho/PXL=25/71/1/3, 10 mM lipids, and (PXL-PEG) is DOTAP/DOPC/DOPE-Rho/DOPE-PEG/PXL=25/66/1/5/3, 10 mM lipids. Data are obtained from three repetitions of donor liposomes incubated with non-radioactive acceptor POPC liposome (10-50 mM lipids), and presented as average ± RSD from three repetitions.*

[Acc]/([Don] + [Acc])	$[PXL]_{Th}$ (µM)	$[PXL]_{Acc}$ norm (µM)	$[PXL\text{-}PEG]_{Acc}$ norm (µM)
0	0	--	--
0.5	2.00	1.12 ± 0.13	0.67 ± 0.05
0.67	2.80	1.46 ± 0.28	1.87 ± 0.24
0.75	3.25	2.08 ± 0.42	2.11 ± 0.96
0.8	3.50	1.96 ± 0.30	1.60 ± 0.22
0.83	3.67	2.39 ± 0.58	1.58 ± 0.57

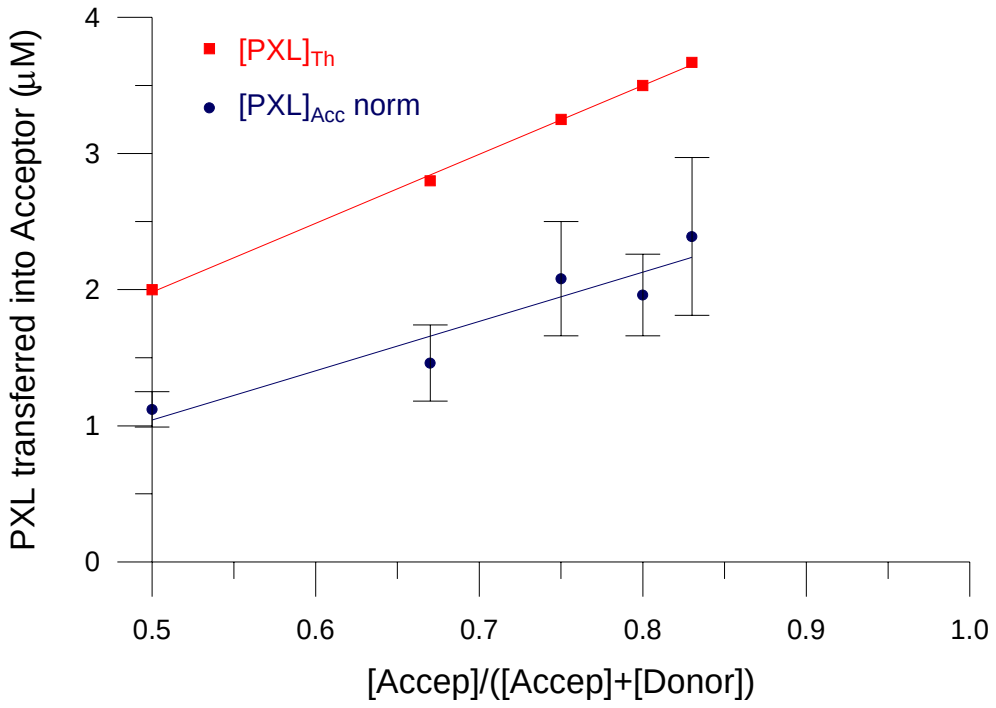

Figure 6.8: *Theoretical and experimental values after normalization of PXL transfer (Table 6.14) from donor(PXL) liposomes into non-radioactive acceptor POPC liposomes after 30 min's incubation against the ratio of acceptor liposome concentration to donor and acceptor liposome concentration. Results were measured by HPLC and error bars are calculated from three repetitions.*

As shown in Figure 6.8, the experimental data of paclitaxel transferred from donor (PXL) into acceptor can be fitted to a linear curve within the limits of error ($y = 3.5258x - 0.7113$, $r^2 = 0.8616$), though the experimental values obtained are relatively smaller than the theoretical ones. In fact, the actual total amount of paclitaxel in the transfer experiment is smaller than 5 µM, the recovery of acceptor liposomes from each incubation is not exactly 89 % and a certain amount of donor liposomes are recovered in the eluent due to the limitation of the ion-exchange columns. All these factors might contribute to explain the difference of experimental results from the theoretical values.

Figure 6.9 shows the linear fits of theoretical values and the experimental values after normalization of the PXL transfer from donor (PXL-PEG) liposomes (lipid composition: DOTAP/DOPC/DOPE-Rho/DOPE-PEG/PXL in a molar ratio of 25/66/1/5/3) into acceptor POPC liposomes against the ratio of acceptor concentration to the sum of donor and acceptor concentration.

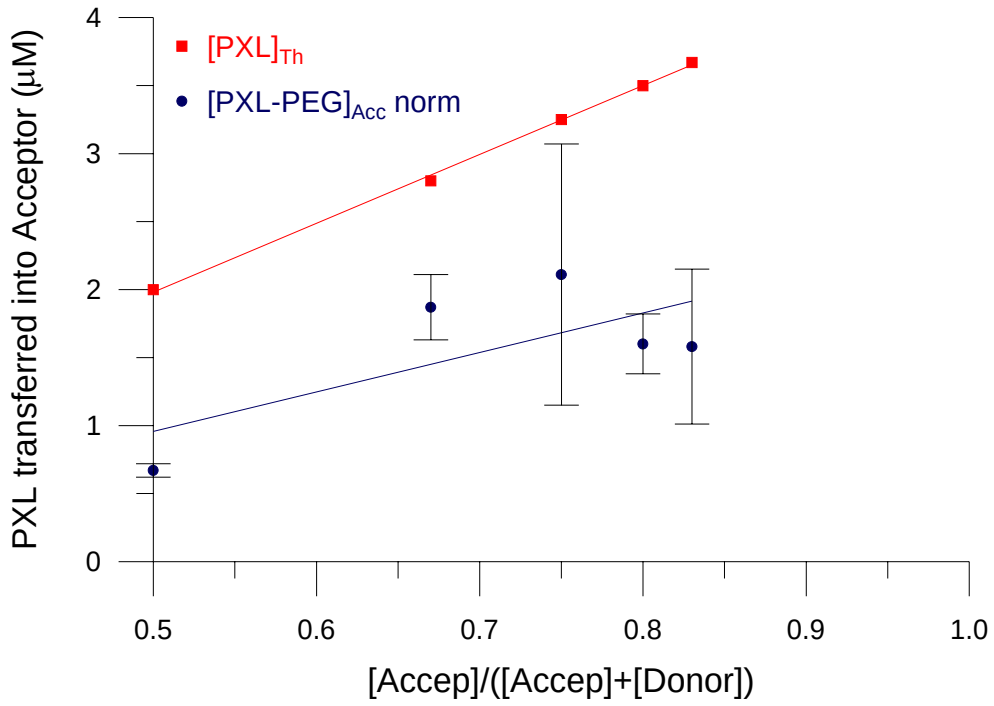

Figure 6.9: *Theoretical and experimental values after normalization of PXL transfer according to Table 6.14 from donor (PXL-PEG) liposomes into acceptor POPC liposomes at 30 min incubation time against the ratio of acceptor liposome concentration to donor and acceptor liposome concentration. Results were measured by HPLC and error bars are calculated from three repetitions.*

As shown in Figure 6.9, the experimental values of the paclitaxel transfer from donor (PXL-PEG) liposomes to acceptor liposomes are also smaller than the theoretical values. The

reasons are similar as the explanation for donor (PXL) liposomal formulation. The results of paclitaxel transfer for acceptor lipids concentrations of 20 and 30 mM are higher than the results of paclitaxel transfer for acceptor lipids concentrations of 40 and 50 mM. In this case, the least squares analysis shows an even lower value of r^2 ($y = 2.7221x - 0.4727$, $r^2 = 0.6649$) together with a higher dispersion of the results. As discussed already in Figure 6.6, more than 15 % of the donor (PXL-PEG) liposomes are recovered in the eluent after being separated by the ion-exchange micro-column. This value increases to nearly 20 % for the acceptor lipids concentration of 50 mM. The donor (PXL-PEG) liposomes have a much lower zeta-potential, of only +24.8 mV, so that this inadequate positive charge is the main reason for the worse donor-acceptor separation. As it can be seen in the Figure 6.9, the error bars of the paclitaxel transfer for acceptor lipid concentration of 30 and 50 mM are very big. All these factors might be the reason for the less efficient separation of donor and acceptor liposomes by the ion-exchange micro-columns.

Due to the low zeta-potential of the donor (PXL-PEG) liposomal formulation, the donor liposomes could not be separated effectively from the acceptor liposomes by the ion-exchange micro-columns. The experimental design for donor (PXL-PEG) is not as successful as the one for donor (PXL) liposomal formulation. In order to get a better comprehension of the donor (PXL-PEG) liposomes, it would be necessary to increase in the future steps, the DOTAP concentration to ensure a sufficient zeta-potential as well as to decrease the liposome application amount onto the ion-exchange micro-columns.

6.4.2 Paclitaxel transfer kinetics from two types of donor to acceptor liposomes

The transfer kinetics of paclitaxel from donor into acceptor liposomes during 30 min incubation was performed by measuring the paclitaxel transfer at different time points from the incubation of donor (PXL) with acceptor liposomes or donor (PXL-PEG) with acceptor liposomes. The donor (PXL) liposomes have lipid composition of DOTAP/DOPC/DOPE-Rho/PXL in a molar ratio of 25/71/1/3, lipid concentration of 10 mM. The donor (PXL-PEG) liposomes have lipid composition of DOTAP/DOPC/DOPE-Rho/DOPE-PEG/PXL in a molar ratio of 25/66/1/5/3, lipid concentration of 10 mM. Both non-radioactive POPC liposomes and radioactive POPC/[^{14}C] CO liposomes with the lipid concentration of 50 mM were prepared as acceptor liposomes. Two incubation groups were designed as I) Donor:Water = 1:1 (v/v) and II) Donor (10 mM):Acceptor (50 mM) = 1:1 (v/v). At the selected time points of 0.5 min, 2 min, 5 min, 10 min, 15 min, 30 min, 50µL of the suspensions were applied on the columns and eluted by 1.5 mL water; with three repetitions at each time point. The recovery of donor

liposomes was determined by fluorescence spectroscopy and the data were calculated from six repetitions (three times incubation of donor with non-radioactive POPC acceptor and three times incubation of donor with radioactive POPC [^{14}C]CO acceptor). The recovery of radioactive acceptor liposomes was determined as the recovery of [^{14}C] CO by LSC, from the three donor with non-radioactive POPC acceptor incubations. The transfer amount of paclitaxel was measured by HPLC/UV, from the three incubations of donor and non-radioactive acceptor liposomes. The results of donor recovery of donor (PXL) and donor (PXL-PEG) in the eluent after separated by the ion-exchange micro-columns during 30 min incubation are gathered in Table 6.15.

Table 6.15: *Recovery of donor (PXL) and donor (PXL-PEG) liposomes calculated by Rho fluorescence measurement from donor lipids in eluent at different incubation times. Results are obtained from six repetitions. Three from incubation of donor and radio-labelled acceptor POPC/[^{14}C] CO liposomes, three from incubation of donor and non-radio-labelled acceptor POPC liposomes. The results are presented as average ± RSD, and calculated from these six repetitions.*

Incubation time (min)	Don. recovery (%) Don. (PXL) + Acc.	Don. recovery (%) Don. (PXL-PEG) + Acc.
0.5	10.37 ± 34.61 %	16.86 ± 34.22 %
2	9.85 ± 28.12 %	19.12 ± 31.74 %
5	9.00 ± 46.22 %	16.79 ± 25.31 %
10	9.92 ± 53.12 %	20.02 ± 34.21 %
15	9.87 ± 34.34 %	20.99 ± 31.82 %
30	8.50 ± 24.70 %	17.91 ± 26.29 %

An schematic graph of donor liposome recovery from the incubation of donor (PXL) liposomes with acceptor liposomes and donor (PXL-PEG) liposomes with acceptor liposomes (both POPC and POPC/[^{14}C] CO) at selected incubation time is illustrated in Figure 6.10.

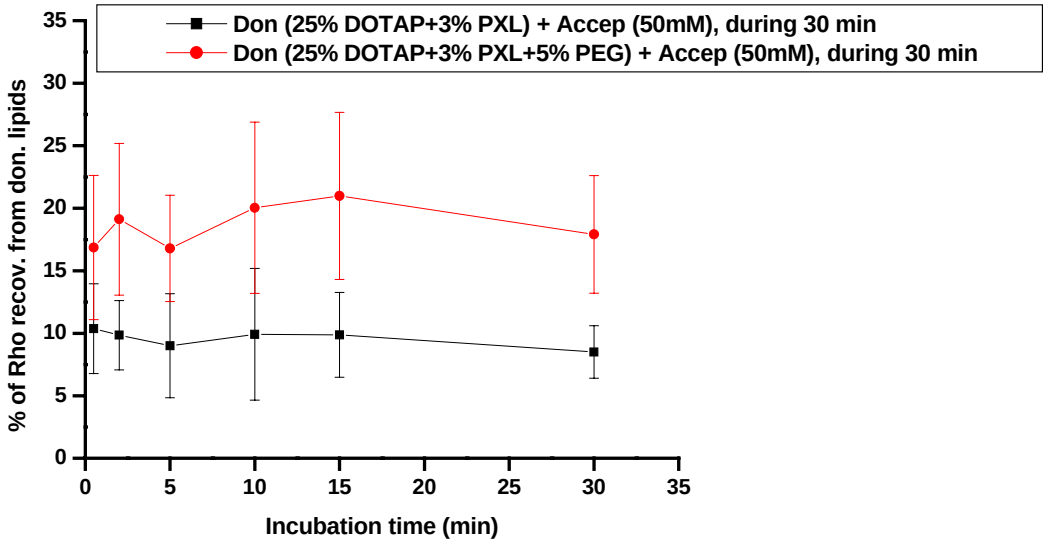

Figure 6.10: *Recovery of donor (PXL) and donor (PXL-PEG) liposomes (see Table 6.15) calculated by rhodamine fluorescence from donor lipids in eluent against incubation time. Error bar is calculated from 6 repetitions. The lines are drawn as a guide for the eyes.*

As seen in Figure 6.10, a constant recovery of both types of donor liposomes in the eluent is observed during 30 min incubation. The recovery of donor (PXL) is constant at around 9 %, and the recovery of donor (PXL-PEG) is round 19 %. The values are in accordance with the results obtained in Figure 6.6 when donor (PXL) or donor (PXL-PEG) is incubated with acceptor (50 mM) after 30 min incubation. As explained in Section 6.4.1, the high donor (PXL-PEG) recovery is due to the low zeta-potential of donor (PXL-PEG) liposomes. Despite the unsuccessful experimental design in donor (PXL-PEG) and acceptor incubation, the transfer of paclitaxel from donor (PXL-PEG) to acceptor liposomes was still examined.

The recovery of radioactive acceptor POPC/[^{14}C] CO liposomes was measured by LSC. The recovery of non-radioactive labelled POPC liposomes is assumed to be comparable with the recovery of radioactive acceptor liposomes. The acceptor POPC [^{14}C] CO liposome recovery at different incubation times from the incubation of donor (PXL) with acceptor liposomes and the incubation of donor (PXL-PEG) with acceptor liopsomes is gathered in Table 6.16.

The acceptor recovery from the incubation of donor (PXL) liposomes (DOTAP/DOPC/DOPE-Rho/PXL=25/71/1/3, lipid concentration 10 mM) with acceptor POPC [^{14}C] CO liposomes and the incubation of donor (PXL-PEG) liposomes (DOTAP/DOPC/DOPE-Rho/DOPE-PEG/PXL=25/66/1/5/3, lipid concentration 10 mM) with acceptor POPC [^{14}C] CO liposomes against incubation time is illustrated in Figure 6.11.

Table 6.16: *Acceptor liposome recovery calculated by [^{14}C]CO radioactivity counting in eluent at different incubation time. The data are obtained from the incubation of donor (PXL) with acceptor POPC [^{14}C]CO liposomes and donor (PXL-PEG) with acceptor POPC [^{14}C]CO liposomes. The results are presented as average ± RSD, and are calculated three repetitions.*

Incubation time (min)	Acc. recovery (%) Don. (PXL) + Acc.	Acc. recovery (%) Don. (PXL-PEG) + Acc.
0.5	89.52 ± 4.70 %	90.17 ± 5.48 %
2	90.01 ± 4.08 %	85.48 ± 6.83 %
5	88.40 ± 5.50 %	89.65 ± 4.55 %
10	89.24 ± 4.32 %	87.81 ± 6.42 %
15	91.25 ± 4.71 %	90.46 ± 4.85 %
30	90.36 ± 4.32 %	89.54 ± 5.42 %

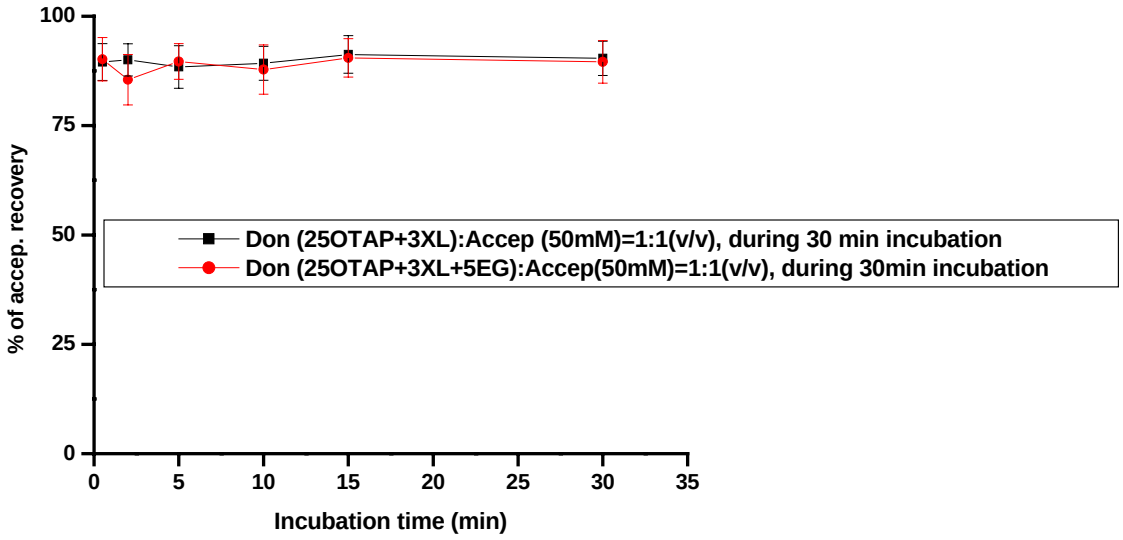

Figure 6.11: *Schematic graph of overlay of acceptor liposome recovery in eluent calculated by [^{14}C]CO radioactivity against incubation time according to Table 6.16. Error bars are calculated from three times incubation of donor with radio-labelled acceptor liposomes. Donor with non-radio-labelled acceptor liposomes was not measured. The lines are drawn as a guide for the eyes.*

The recovery of acceptor liposomes in the eluent from both incubation groups as shown in Figure 6.11 is constant within the range from 85 to 91 %. The type of donor liposomes, the insufficient zeta-potential of donor (PXL-PEG) liposomes and the incubation time do not

produce any significant influence on the recovery of acceptor liposomes. In all the incubation groups, the acceptor liposomes can be separated from donor liposomes in the required manner. As it can be concluded from the results in Tables 6.15 and 6.16, the separation efficiency of the ion-exchange micro-column for donor (PXL) and acceptor liposomes is around 9 % donor recovery and 89 % acceptor recovery. The separation efficiency for donor (PXL-PEG) and acceptor liposomes is around 19 % donor recovery and 89 % acceptor recovery. The high donor (PXL-PEG) recovery might influence the reliability of the paclitaxel transfer results.

The quantification of paclitaxel transferred from donor to non-radioactive acceptor liposomes during 30 min incubation time was measured by HPLC/UV. The obtained results were normalized by assuming a constant acceptor recovery of 89 % in the eluent. The results are presented as the percentage of paclitaxel transfer, which was calculated as the concentration of paclitaxel transferred into acceptor liposomes divided by the total paclitaxel concentration. The detailed calculations are explained in Appendix 3. The data of the percentage of paclitaxel transfer from donor (PXL) and donor (PXL-PEG) liposomes to acceptor liposomes during 30 min incubation are presented in Table 6.17.

Table 6.17: *Paclitaxel percentage transfer from donor (PXL) liposomes and donor (PXL-PEG) liposomes to acceptor POPC liposomes at selected time points during 30 min incubation time. The results are presented as average ± RSD, and are calculated from three repetitions.*

Incubation time (min)	PXL transfer (%) Don (PXL) + Accep	PXL transfer (%) Don (PXL-PEG) + Accep
0	0	0
0.5	57.64 ± 14.15 %	37.05 ± 51.37 %
2	76.94 ± 16.95 %	67.99 ± 50.85 %
5	74.53 ± 15.69 %	67.17 ± 40.62 %
10	78.02 ± 3.89 %	62.49 ± 12.89 %
15	77.75 ± 9.30 %	59.44 ± 18.41 %
30	75.07 ± 15.39 %	53.19 ± 7.20 %

According to the data gathered in Table 6.17, the schematic graph of the paclitaxel transfer kinetics from donor (PXL) liposomes to acceptor POPC liposomes during 30 min incubation fitted by Origin 6.0 software is illustrated in Figure 6.12.

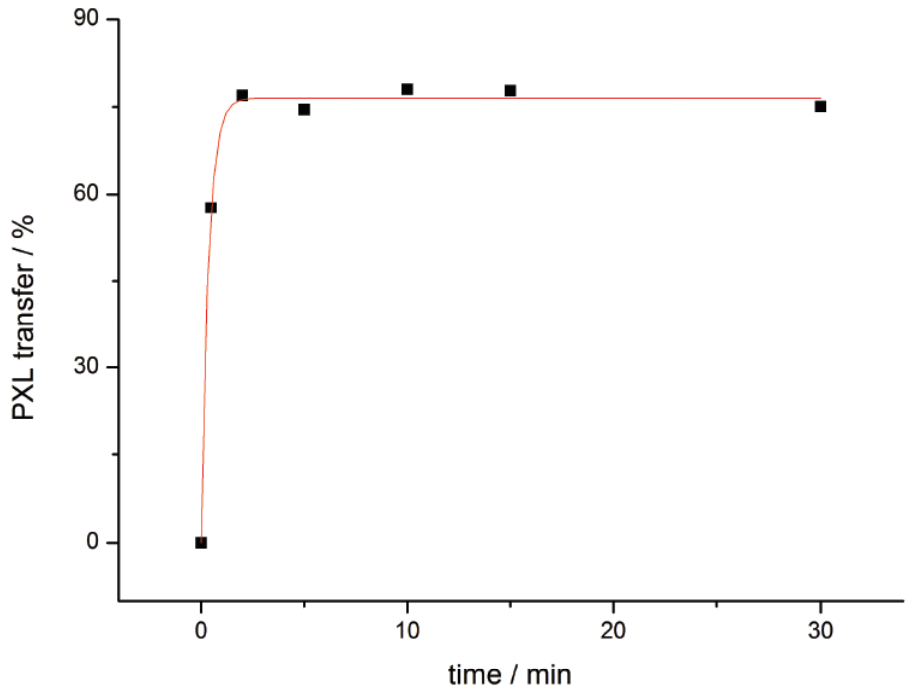

Figure 6.12: *Percentage of paclitaxel transferred from donor (PXL) liposomes to acceptor POPC liposomes during 30 min incubation presented as the transfer percentage (see Table 6.17). The data are fitted into an exponential model* $y = A(1-e^{-t/tau})$. *The time constant of the process was 0.35 ± 0.02 minutes.*

As shown in Figure 6.12, at 30 seconds incubation, around 57 % of paclitaxel is already transferred into acceptor liposomes, within the first two minutes the transfer amount of paclitaxel reaches more than 77 %. Then, the percentage transfer of paclitaxel remains constant at around 78 % within the limits of error during the whole incubation time. The expected equilibrium transfer of the drug in the experiments is 83.3 % since the lipids concentration of acceptor vesicles are 5-times in excess. The experimental values obtained under the conditions of the ion-exchange micro-column model are comparable with the theoretical value. The transfer of paclitaxel occurs in an instantaneous way after the incubation, and paclitaxel is equally redistributed between DOPC donor and POPC acceptor liposomes. The behaviour of paclitaxel transfer from donor (PXL) to acceptor liposomes can be adjusted to an exponential model as illustrated in Figure 6.12. The exponential model can be described as y = A + (1 - exp(-t/tau)). The coefficient of regression of the least squares analysis is $r^2 = 0.952$. The tau-values are describing the time constant of the transfer process.
In accordance with the results in Table 6.17, the schematic graph of the paclitaxel transfer kinetics from donor (PXL-PEG) liposomes to acceptor POPC liposomes during 30 min incubation fitted by Origin 6.0 software is illustrated in Figure 6.13.

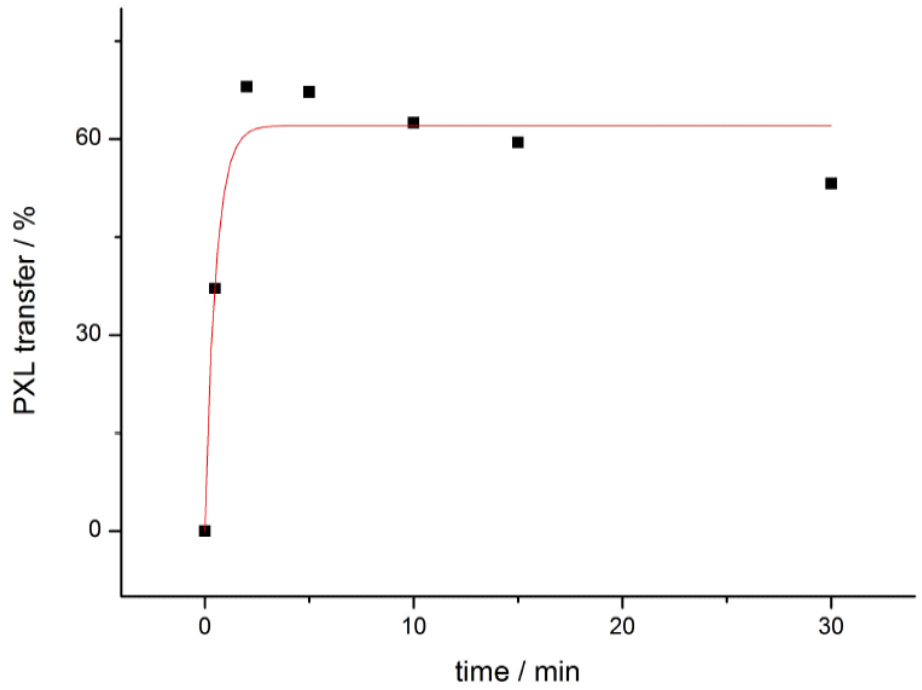

Figure 6.13: *Paclitaxel transfer kinetics from donor (PXL-PEG) liposomes to acceptor POPC liposomes during 30 min incubation time, presented as the transfer percentage (see table 6.17). The data are fitted into an exponential model. The time constant of the process was 0.50 ± 0.13 minutes.*

As it can be seen in Figure 6.13, the transfer amount of paclitaxel from donor (PXL-PEG) liposomal formulation during 30 minutes incubation is generally smaller compared to the one from donor (PXL) liposomal formulation. Only around 37 % of paclitaxel is transferred into acceptor liposomes at 30 seconds incubation and the transfer amount of paclitaxel reaches more than 67 % within the first two minutes incubation. However, a decreasing trend of the paclitaxel percentage transfer from 67 % to 53 % is observed during 5 to 30 min incubation period. It is worth to mention that, the RSD values at the first three incubation time points are much larger compared to those at the last three incubation time points. The experimental points are fitted also to an exponential model, and the coefficient of regression of the least square analysis is $r^2 = 0.641$. The r^2 value indicates that the exponential fitting of the transfer of paclitaxel from donor (PXL-PEG) liposomes to acceptor liposomes is not as reliable as the transfer of paclitaxel from donor (PXL-PEG) to acceptor liposomes. Some reasons might be responsible for the high RSD values: on one hand, as it has been proved in Section 6.3.3 and Section 6.4.1, the zeta-potential plays an important role in the successful separation by the ion-exchange micro-columns. The donor (PXL-PEG) formulation has the zeta-potential of only +24.8 mV. With such a low zeta-potential, the donor liposomes do not have a strong

interaction with the ion-exchange gel, therefore, it is not surprising to observe that in Figure 6.10, nearly 20 % of the donor liposomes are recovered in the eluent together with the acceptor liposomes. The high RSD values correspond to the low separation efficiency of the ion-exchange micro-columns. On the other hand, the transfer of paclitaxel is still very fast even with the incorporation of PEG, 37 % of paclitaxel is transferred at 30 seconds incubation time and the transfer starts to reach a stable plateau within 2 minutes incubation time. The transfer is quite variable only during the first five minutes incubation time, and the transfer of paclitaxel stops increasing after five minutes incubation. The high RSD values are also an indication of the rapid transfer kinetics within the first few minutes incubation time. After 5 minutes the transfer of paclitaxel is finished, and therefore, the RSD values become smaller and more stable. But it is arbitrary to accept that the decreased transfer of paclitaxel during 5 to 30 minutes incubation time is true, due to the inefficiency of the separation of donor from acceptor liposomes by ion-exchange micro-columns at a low donor zeta-potential.

The generally lower transfer amount of paclitaxel from donor (PXL-PEG) liposomes to acceptor liposomes during 30 minutes incubation can be explained as follows: in the donor (PXL-PEG) liposomal formulations, the outer layer of the lipid bilayer is surrounded by the big head groups of PEG due to the incorporation of PEG. Some amount of the paclitaxel might transfer into the big head groups of the PEG instead of the bilayer of acceptor liposomes. Then, the transfer amount of paclitaxel from donor (PXL-PEG) to acceptor liposomes is generally lower than the transfer amount of paclitaxel from donor (PXL) liposomes, though the transfer rate is quite comparable in both of the donor liposomal formulations.

Summary

In the linear relation of paclitaxel transfer experiments, a linear curve is obtained from the donor (PXL) formulation, even though the values are relatively smaller than the theoretical ones. Therefore, the results are considered to be acceptable under the experimental conditions used. The linear curve obtained in the case of paclitaxel transfer from donor (PXL-PEG) formulation is, however, not taken with the same value due to the insufficient zeta-potential of donor (PXL-PEG).

In the paclitaxel transfer kinetics experiment, an instantaneous transfer of paclitaxel between lipid bilayer membranes is observed in both donor (PXL) and donor (PXL-PEG) formulations. In donor (PXL) formulation, around 57 % of paclitaxel is transferred at 30 seconds incubation time, and a plateau of around 78 % transfer of paclitaxel is obtained after

2 minutes incubation time. The maximal paclitaxel transfer is quite comparable with the expected value of 83 %. PXL might be equally distributed between donor DOPC liposome and acceptor POPC liposome membranes. For the donor (PXL) formulation, the transfer kinetics can be fitted with a good accordance into an exponential model. In the case of paclitaxel transfer from donor (PXL-PEG) formulation, large RSD values and low r^2 value indicate a lower reliability of the experimental design. Due to the low zeta-potential of donor (PXL-PEG) liposomes, the separation efficiency of donor from acceptor liposomes by the ion-exchange micro-columns is seriously deteriorated. A large amount of donor liposomes are detected in the eluent, which influences in a great extent an accurate quantification of paclitaxel transfer. Further comparison of paclitaxel transfer, from these two types of donor liposomal formulations, and the evaluation of the effect of PEG in the paclitaxel transfer are not easy to carry out due to the drawbacks in the donor (PXL-PEG) experimental designs.

The incorporation of PEG gives rise to a decreased paclitaxel transfer amount, but it does not influence the transfer rate. Due to the steric structure of PEG, some amount of paclitaxel might transfer into the big head groups of the PEG instead of the bilayer of acceptor liposomes.

An instantaneous transfer of paclitaxel between liposomal membranes can be concluded from the obtained data despite the weakness in the donor (PXL-PEG) experimental design. There are several possible explanations for the fast paclitaxel transfer between liposomal membranes. The log P of paclitaxel calculated by the Bio-Loom Windows Software Version 1.0 is 4.73, and a log P value of 3.96 is reported by Hanai T. and co-workers [Hanai T., et al., (2000)]. The log P value indicates a lipophilic character of paclitaxel. It was reported that paclitaxel is miscible in the lipid but with a repulsive interaction between the paclitaxel and the lipid [Feng S.S. et al., (2002)]. The location of paclitaxel in the bilayer might be in the outer hydrophobic cooperative zone of the bilayer, i.e., in the region of the C1-C8 carbon atoms of the acyl chain, or just remained adsorbed on the polar head group layer of the phospholipid. Such a location for paclitaxel in the bilayer could be anticipated from the structure of the drug. The C13 side chain of the paclitaxel (see Figure 6.14) is relatively more hydrophobic because of the two aromatic rings, while the main taxane ring bears substituents that have comparatively greater propensity for polar interactions. However, the drug may intrude into the inner hydrophobic zone of the lipid bilayer depending on the different lipid types (lipid chain unsaturation, head-group types, etc). Furthermore, the paclitaxel molecule does not have typical amphiphilic properties. It cannot be in good array in the lipid bilayers. Compared with the chain-headgroup structure of lipid molecules, paclitaxel molecules are

quite bulky. The lipid molecules are loosely packed and the hydrophobic tail groups are tilted towards the interface. The insertion of paclitaxel into the lipid molecules would take more space and push the lipid molecule to be closer to each other. But at the same time, the intermolecular force would try to resist the insertion of paclitaxel into the lipid bilayer. This intermolecular force increases with more paclitaxel incorporated into the lipid bilayer. As a result of the tendency of reducing this intermolecular force of the lipids, paclitaxel is forced to leave the donor lipids and to be redistributed into acceptor lipid membranes. That can be used to explain why paclitaxel is transferred in a really fast way between liposomal membranes [Zhao L.Y. et al., (2004); Zhao L.Y. and Feng S.S., (2004); Zhao L.Y. and Feng S.S., (2005)].

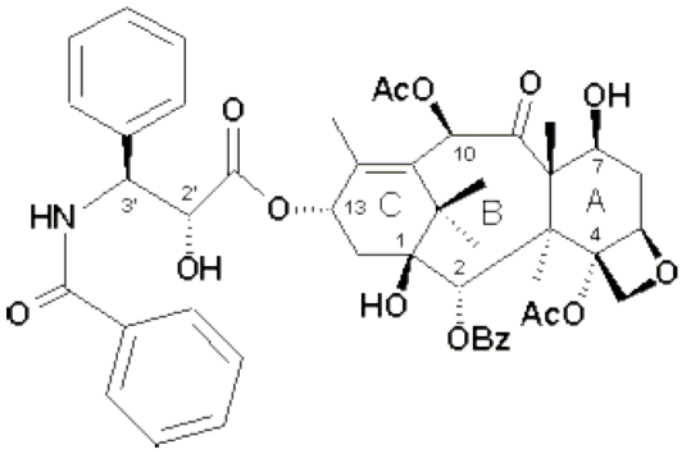

Figure 6.14: *Molecular structure of paclitaxel*

6.5 Cholesterol transfer between liposomal membranes

Cholesterol transfer from both positively and negatively charged donor liposomes to acceptor liposomes was studied as a comparison with paclitaxel transfer and the influence of DOTAP to cholesterol transfer was examined. The composition of positively charged cholesterol-containing liposomes was DOTAP/DOPC/Chol/[^{3}H]Chol in the molar ratio of 25/72/3. The composition of negatively charged cholesterol-containing liposomes was DCP/POPC/Chol/[^{3}H]Chol in the molar ratio of 1/7/2. The acceptor liposomes were prepared as POPC/Chol/[^{14}C] CO in the molar ratio of 97/3 for incubating with positively charged donor liposomes and POPC/Chol/[^{14}C] CO in the molar ratio of 8/2 for incubating with negatively charged donor liposomes. The lipid concentration of both donor and acceptor liposomes was 10 mM. Each incubation was repeated three times. Three incubation groups were designed as I) Donor:Water = 1:5 (v/v), II) Water:Acceptor = 1:5 (v/v) and III) Donor:Acceptor = 1:5 (v/v). The transfer study was performed by incubating donor with

acceptor in a volume ratio of 1 to 5, from the incubation mixture, 10 µL of the mixture were taken and separated in the ion-exchange micro-columns. The transfer of cholesterol at selected incubation time points during 24 h incubation was measured by LSC.

The transfer of cholesterol (Chol) from both positive and negative donor liposomes to acceptor liposomes during a 24 hours incubation was quantified by [^{3}H] Chol measurement and the recovery of acceptor liposomes was controlled by [^{14}C] CO measurement. The transfer of cholesterol at each time point was normalized to 100 % according to the respective acceptor recovery (see Section 5.2 for calculation). The percentages transfer from both positively and negatively charged donor liposomes to acceptor liposomes at different incubation times are gathered in Table 6.18.

Table 6.18: *Transfer kinetics of cholesterol from positively charged donor (Chol) liposomes and negatively charged donor (Chol) liposomes to acceptor liposomes during 30 min incubation time. The results are presented as average ± RSD, and calculated from three repetitions.*

Incubation time (min)	Positive Chol transfer (%)	Negative Chol transfer (%)
0	0	0
2	37.30 ± 4.82 %	4.40 ± 33.18 %
5	40.81 ± 9.85 %	7.59 ± 39.13 %
10	44.17 ± 7.35 %	11.27 ± 27.11 %
15	46.03 ± 8.51 %	17.74 ± 12.96 %
30	49.49 ± 14.43 %	31.88 ± 12.86 %
60	55.41 ±4.42 %	53.91 ± 8.27 %
120	65.72 ± 6.61 %	65.71 ± 11.53 %
240	77.85 ± 7.88 %	76.97 ± 2.84 %
1440	79.83 ± 3.11 %	81.53 ± 3.55 %

According to the data shown in Table 6.18, the schematic graph of cholesterol transfer kinetics from positive donor (Chol) liposomes to acceptor liposomes during 24 h incubation fitted by Origin 6.0 into an exponential model is illustrated in Figure 6.15.

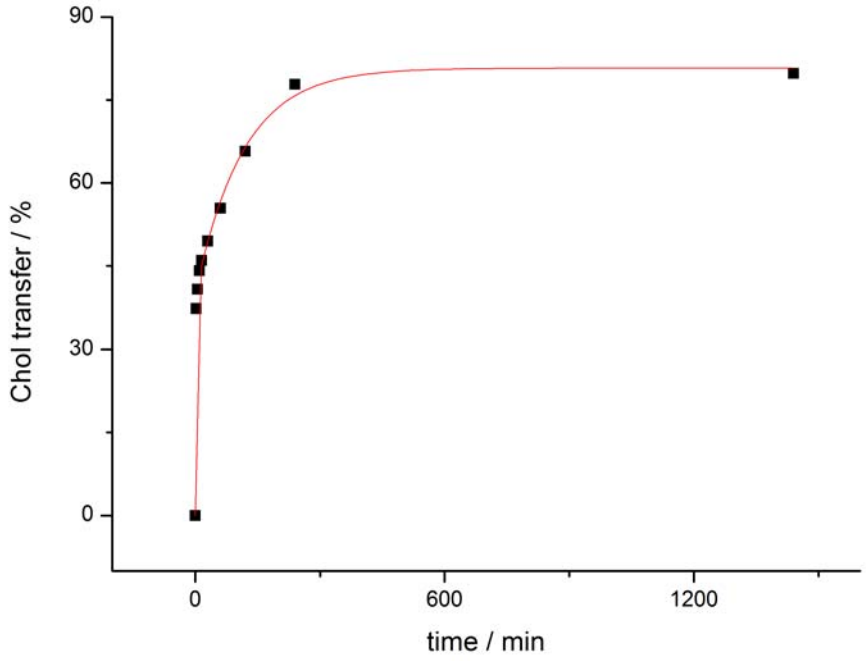

Figure 6.15: *Transfer kinetics of Chol from positively charged donor (Chol) liposomes to acceptor liposomes during 24 h incubation according to Table 6.18, presented in %. The slow transfer process is characterized by a time constant of 114 ± 10 min.*

As shown in the Figure 6.15, there is a rapid transfer of cholesterol during the first 15 minutes of incubation. The 46 % of cholesterol is already transferred into acceptor liposomes at 15 minutes incubation time. The transfer speed slows down noticeably after 15 minutes. After the following 15 minutes, only 3 % cholesterol is further transferred into acceptor liposomes. After 120 minutes, up to 65 % of cholesterol is transferred into acceptor. The equilibrium transfer is nearly 80 % after 24 hours incubation. The data of cholesterol transfer are fitted by using the exponential model. The transfer of cholesterol from positively charged donor liposomes to acceptor liposomes shows a two-phase transfer process, a fast transfer process and a slow transfer process. The slow transfer process is characterized by a time constant of around 114 min. The incorporation of DOTAP deteriorates the retention of cholesterol in the donor liposomes, therefore, a fast release of cholesterol from donor liposomes is observed. But this effect influences only partially the cholesterol transfer. The transfer speed tends to slow down after 50 % of the cholesterol is transferred into acceptor.

Figure 6.16 shows the cholesterol transfer kinetics from negative donor (Chol) liposomes to acceptor liposomes during 30 min incubation, according to the data presented in Table 6.18.

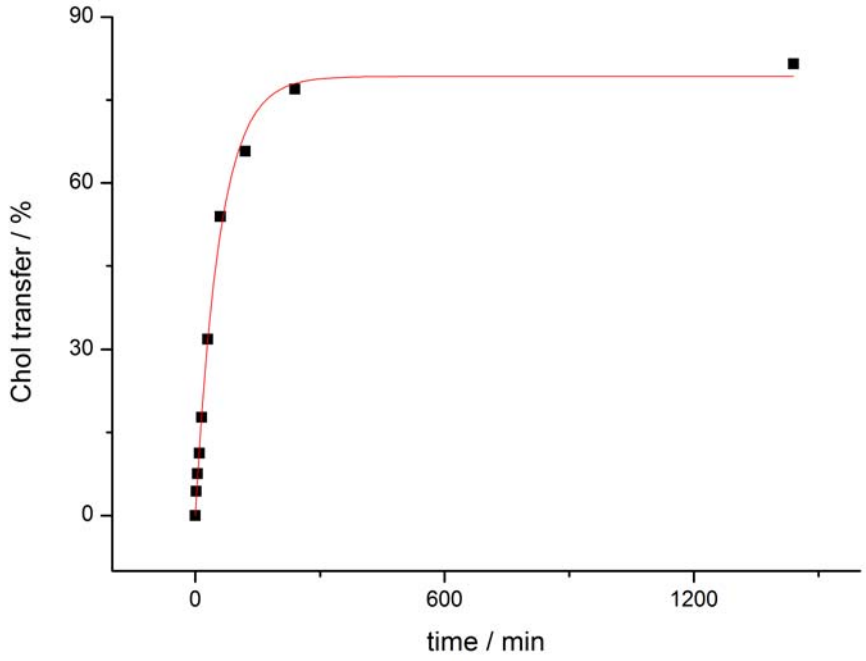

Figure 6.16: *Transfer kinetics of cholesterol from negatively charged donor (Chol) liposomes to acceptor liposomes during 24 h incubation according to Table 6.18, presented in %.The transfer process is characterized by a time constant of 58.2 ± 3.2 minutes.*

The percentage transfer of cholesterol in the first 15 minutes is much slower compared to the fast transfer of cholesterol from positively charged donor (Chol) liposomes. The transfer of cholesterol keeps on increasing up to around 53 % during the first 60 minutes of incubation. The transfer behaviour of cholesterol from negatively charged donor (Chol) liposomes is quite comparable with the transfer behaviour of positively charged donor (Chol) liposomes during the incubation from 1 until 24 hours. The data are fitted also into a single exponential model. Unlike cholesterol transfer from positively charged donor (Chol) liposomes, only one phase transfer process is observed in the case of negatively charged donor (Chol) formulation, with a time constant of around 58.2 minutes.

Summary

Around 80 % of cholesterol is transferred into the acceptor liposomes from both positively and negatively charged donor liposomes. According to the expected 83.3 % equilibrium transfer of the drug, an equal distribution of cholesterol between donor and acceptor liposomal membranes is fulfilled.

The composition of positively charged cholesterol-containing donor liposomal formulation is DOTAP/DOPC/Chol/[^{3}H]Chol in a molar ratio of 25/72/3, and the composition of negatively charged cholesterol containing donor liposomal formulation is DCP/DOPC/Chol/[^{3}H]Chol in a molar ratio of 2/7/1. The radio-labelled cholesterol: [1α,2α(n)-^{3}H]Cholesterol ([^{3}H] Chol) is used for the transfer purpose. The final concentration of [1α,2α(n)-^{3}H]Cholesterol in both formulations is 1 μCi/ml. The non-radioactive cholesterol serves to improve the lipids quality. However, the cholesterol concentration of the two formulations is not comparable. The cholesterol molar contain in positive donor (Chol) liposomes is 3 mol %, and in negative donor (Chol) liposomes is up to 10 %. The difference in cholesterol content might influence the [1α,2α(n)-^{3}H]Cholesterol transfer. For a convincing comparison of [1α,2α(n)-^{3}H]Cholesterol transfer between positively charged or negatively charged donor liposomes, it is recommended to prepare the liposomes with the same cholesterol content. Despite this shortcoming in the study, the transfer of cholesterol kinetics from the two types of donor (Chol) liposomes is compared. And some interesting aspects of the transfer kinetics of cholesterol are discovered: The time constant of cholesterol transferred from negatively charged donor into acceptor liposomes is found to be around 58 min, which is quite comparable with a similar work done by Fahr A. and co-wokers [Fahr A. and Seelig J., (2001)], where a transfer half time of 60 min was obtained. The transfer process of cholesterol form positively charged donor into acceptor liposomes is found to consist of two phases: a rapid phase and a slow phase. The slow transfer process is characterized by a time constant of around 114 minutes. These differences in the transfer kinetics of cholesterol between the positive and negative donor (Chol) liposomes are induced by the incorporation of DOTAP. Therefore, the presence of DOTAP gives rise to a deteriorated retention of cholesterol in the positively charged donor liposomes, at least during the first 30 minutes incubation. As it is known, the DOTAP lipid molecular has some distinguished characteristics compared to the other lipids. The unsaturated acyl chains and the charged head groups of DOTAP are supposed to influence the lipid membrane fluidity to a great extent [Campbell R.B. et al, (2001)]. The incorporation of DOTAP increases the lipid membrane fluidity, which therefore, facilitates the transfer of cholesterol between the liposomal membranes. All these characters might be accountable for the fast release of cholesterol from positively charged donor (Chol) liposomes during the first 30 minutes incubation.

The possible mechanisms of cholesterol transfer have been investigated by many researchers. Haran N. and Shporer M. have ruled out fusion as a prerequisite for cholesterol exchange by using Nuclear Magnetic Resonance experiments [Haran N. and Shporer M., (1977)]. In a

simple model system which is not influenced by fusion or the presence of proteins, two limiting models for cholesterol exchange must be considered.

One is a process which involves the transitory mixing of the donor and acceptor vesicle bilayers followed by diffusion of lipid molecules within this collision complex [Gurd F.R.N., (1960)]. This mechanism model is usually named as “Collision complex model”. In such a model, either diffusion within the collision complex or collision frequency may be rate limiting, and collisions are blind to the compound transferred as well. [Kuo A.-L. and Wade C.G., (1979); Träuble H. and Sackmann E., (1972)]. In a previous investigation from McLean L.R. and co-workers, the exchange of cholesterol between unilamellar vesicles is a first-order process independent of the concentration of acceptor vesicles over a wide range of concentrations and independent of the incubation temperature [McLean L.R. and Phillips M.C., (1981)]. The observation from McLean L.R. and co-workers that around 80% of the cholesterol in positively or negatively charged vesicles is transferred in a first-order process indicates that transfer from the inner to outer monolayer of the vesicle bilayer is not a rate-limiting step in cholesterol transfer [McLean L.R. and Phillips M.C., (1981)], since ~67% of the cholesterol in unilamellar vesicles resides in the outer monolayer [Huang C.H., (1969)]. A similar results has been obtained by other investigators like Backer J.M. and Nakagawa Y. [Backer J.M. and Dawidowicz E.A., (1979); Nakagawa Y. et al., (1979)]. These observations indicate that the collision complex mechanism is not an adequate description of the cholesterol exchange between unilamellar vesicles.

The other transfer mechanism involves desorption of the compound molecules from the donor bilayer into the aqueous phase where the soluble compound molecules are free to collide with acceptor vesicles [Hagerman J.S. and Gould R.G., (1951)]. This mechanism model is usually named as “Aqueous diffusion model”. Although the molecular mechanism of cholesterol exchange in unilamellar vesicles has not been absolutely defined, fluorescent lipid molecules and phospholipids have shown to exchange via the aqueous phase [Martin F.J. and MacDonald R.C., (1976); Duckwitz-Peterlein G., et al., (1977); Thilo L., (1977); Roseman M.A. and Thompson T.E., (1980)]. Aqueous diffusion may follow either first- or second-order kinetics depending on the rate-limiting step in exchange. First-order kinetics is predicted when desorption is the rate-limiting step. On the other hand, collisions between lipid molecules (presumed to be present in the water as monomers) and the acceptor vesicle in the adsorption step would proceed at a rate proportional to the concentration of acceptor vesicles. As reported by McLean L.R. and co-workers, there is not an obvious evidence for such a prevailing collision mechanism; instead, the kinetics indicates that exchange is

independent of the concentration of acceptor vesicles and that desorption is the rate-limiting step in cholesterol and phospholipid exchange between unilamellar vesicles [McLean L.R. and Phillips M.C., (1981)].

The log P of paclitaxel and cholesterol is around 4.73 and 9.52, respectively, as calculated by Bio-Loom Windows Software Version 1.0. These values are only a theoretical value of the compounds distribution coefficient between octanol and water. The log P value alone could not predict the transfer behaviour of the compounds between the liposomal membranes. The transfer mechanism of one compound should be influenced by many other factors, such as the character of compound molecular, the difference in lipid types. Based on the understanding of these two transfer mechanism model, the transfer speed of a “Collision complex model” dominated transfer should be much faster compared to an “Aqueous diffusion model” dominated transfer. In the case of paclitaxel transfer, around 80 % of the paclitaxel is transferred almost instantaneously within two minutes of incubation, while in the case of cholesterol transfer, the transfer of cholesterol begins to reach a plateau at around 80 % after 240 minutes incubation,. It is assumed that the transfer of paclitaxel prefers the “Collision complex model”, and in the transfer of cholesterol from both positive and negative donor liposomes the transfer behaviour may be dominated by the “Aqueous diffusion model”. Figure 6.17 illustrates one possible explanation of the cholesterol exchange, which is dominated by the “Aqueous diffusion model” process. Cholesterol desorbs in a rate-limiting step from the donor bilayer into the aqueous phase. Desorption does not involve a one by one exchange of lipid at a particular site. The monomeric molecules have no memory of the vesicle from which they arise and collide with any other vesicle in a random process. Following collision, cholesterol is rapidly adsorbed into the acceptor bilayer.

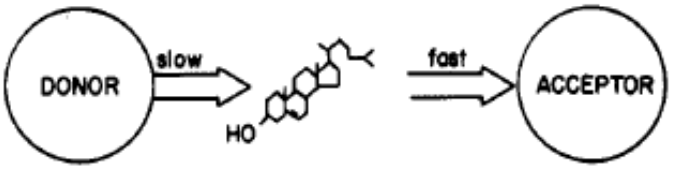

Figure 6.17: *Illustration of the mechanism of cholesterol exchange and transfer between unilamellar vesicles.*

6.6 Cholesteryl-oleoyl-ether transfer between liposomal membranes

The experiments used for the transfer of cholesteryl-oleoyl-ether (COE) from both positively and negatively charged donor liposomes to acceptor liposomes was designed in the same way as the experiment for the transfer of cholesterol. The composition of positively charged COE-containing liposomes is DOTAP/DOPC/Chol/[^{3}H]COE in the molar ratio of 25/72/3. The composition of negatively charged COE-containing liposomes is DCP/POPC/Chol/[^{3}H]COE in the molar ratio of 1/7/2. The acceptor liposomes were prepared as POPC/Chol/[^{14}C] CO in the molar ratio of 97/3 for positively charged donor liposomes and POPC/Chol/[^{14}C] CO in the molar ratio of 8/2 for negatively charged donor liposomes. The lipid concentration of both donor and acceptor liposomes was 10 mM. The transfer of COE was performed by incubation of donor and acceptor with the same lipids concentration in a volume ratio of 1 to 5. 10 µL of the mixture were measured at different incubation time points during a 24 h incubation. Each incubation was repeated three times. The transfer of COE between liposomal membranes during a 24-hour incubation was quantified by [^{3}H] measurement and the recovery of acceptor liposomes was controlled by [^{14}C] measurement. The data of COE transfer from both positively and negatively charged donor liposomes to acceptor liposomes at different incubation time are gathered in Table 6.19.

Table 6.19: *Transfer kinetics of COE from positively charged donor (COE) liposomes to acceptor liposomes during 30 min incubation time. The results are presented as average ± SD, and are calculated from three repetitions.*

Incubation time (min)	Positive COE transfer (%)	Negative COE transfer (%)
0	0	0
2	0	0
5	0	0
10	0	0
15	0	0
30	0	0
60	0	0
120	0.06 ± 0.06	0.02 ± 0.02
240	0.11 ± 0.07	0.06 ± 0.03
1440	0.24 ± 0.05	0.18 ± 0.04

The schematic graph of the overlay of COE transfer kinetics from both positive and negative donor (COE) liposomes to acceptor liposomes during 24 h incubation is illustrated in Figure 6.17.

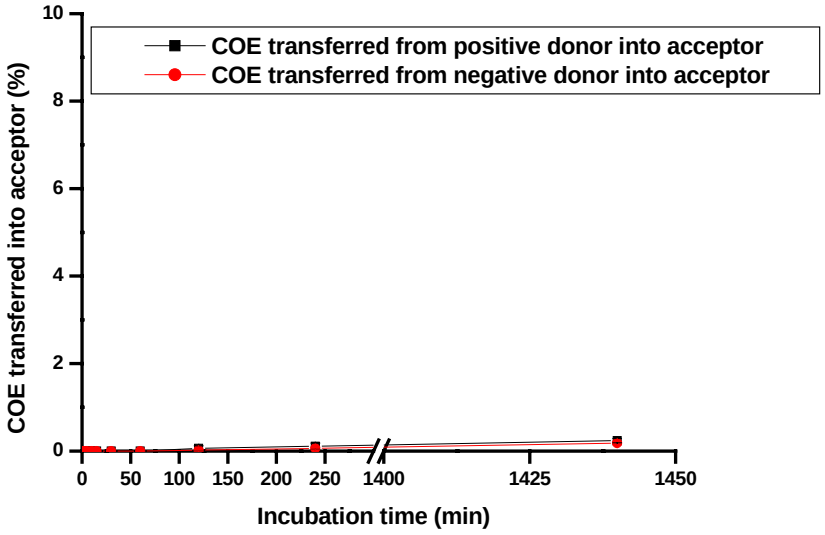

Figure 6.17: *Transfer kinetics of COE from both positively and negatively charged donor (COE) liposomes to acceptor liposomes during 24 h incubation according to Table 6.19, presented in %.*

The transfer of COE from both positive and negative donor liposomes is proved to remain for prolonged periods of time in the host membrane. The time constant for the transfer process of the positively charged membranes was determined to be 353 days; for the negatively charged liposomes a time constant of 485 days was estimated. The molecule of these compounds has a strong affinity with the lipid membranes. A transfer time constant of about 250 days is reported by Fahr A. and co-workers [Fahr A. and Seelig J., (2001)]. As a result of this character, the radioactive label of cholesteryl-oleoyl-ether or cholesteryl oleate is widely used as a non-exchangeable marker for tracing the movement of liposomes [McLean L.R. and Phillips M.C., (1981); Bar L.K. et al., (1987); Fahr A. and Seelig J., (2001)].

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Chapter 7

Temoporfin transfer based on fluorescence dequenching effect

7.1 Liposomal size distribution

Z-average and PDI of all the liposomal formulations were checked by PCS immediately after liposome preparation as a quality control. Table 7.1 gathers the Z-average and PDI values for all the liposomal formulations.

Table 7.1*: Size and PDI results of all the formulations. All the measurements were performed the same day after the liposome preparation and all the values were averages from 3 times of measurement.*

Liposome formulation ([lipids]= 20 mg/mL)	Z-average (nm)	PDI
C16	105.4 ± 7	0.051 ± 0.02
C16_TP	110.1 ± 6	0.051 ± 0.01
C16_TP_P	107.8 ± 3	0.063 ± 0.01
C16_TP_Chol	113.2 ± 1	0.065 ± 0.01
C16_TP_P_Chol	102.3 ± 5	0.066 ± 0.04
C16_TP_DOTAP	111.3 ± 5	0.047 ± 0.01
C18	106.4 ± 5	0.063 ± 0.01
C18_TP	109.7 ± 3	0.074 ± 0.03
C18_TP_P	116.2 ± 7	0.060 ± 0.02
C18_TP_Chol	107.4 ± 3	0.078 ± 0.02
C18_TP_P_Chol	112.1 ± 4	0.059 ± 0.01
Accep.	123.5 ± 3	0.091 ± 0.03

The Z-average of all the donor liposomes is within the range from 102 to 116 nm. The Z-average of acceptor liposomes is around 123 nm, slightly larger than donor liposomes. PDI of all the liposomes were lower than 0.1. The incorporation of temoporfin or other lipids does not show a significant difference in the size of the liposomes. It can be concluded that the parameters meet liposome quality control.

7.2 Differential Scanning Calorimetry

Determination of the location of drugs within liposomes and their interactions with the lipid bilayer is important in liposome characterization. In this study, Differential Scanning Calorimetry (DSC) was used to investigate the interactions of molecules such as cholesterol (Chol), PEG and the lipophilic drug Temoporfin (TP) with the lipid membranes. The DSC measurement of eleven liposomal formulations was performed. The composition of these liposomal formulations is presented as: 1) C16, 2) C16_TP, 3) C16_TP_Chol, 4) C16_TP_P, 5) C16_TP_P_Chol, 6) C16_ DOTAP_TP, 7) C18, 8) C18_TP, 9) C18_TP_Chol, 10) C18_TP_P and 11) C18_TP_P_Chol (see Table 3.2 for composition ratios). The peak temperature (T_m), onset temperature (Onset), enthalpy (ΔH) and peak area (Area) from these liposomal formulations measured by DSC are presented in Table 7.2.

Table 7.2: *Main phase transition temperature (T_m), enthalpy (ΔH), peak onset, and peak area of six C16 and five C18 liposomal formulations measured by DSC. All the results are obtained from the 1st heating run, by using a heating rate of 5 °C/min.*

Liposome formulations	T_m (°C)	ΔH (J/g)	Onset (°C)	Area (mJ)
C16	42.357	0.756	39.765	8.643
C16_TP	36.518	0.619	34.369	7.465
C16_TP_P	38.117	0.601	33.673	7.739
C16_TP_Chol	35.130	0.523	33.541	6.295
C16_TP_P_Chol	36.933	0.543	35.153	6.830
C16_TP_DOTAP	30.728	0.142	27.218	1.726
C18	55.455	1.087	52.417	14.235
C18_TP	51.026	0.926	49.349	10.653
C18_TP_Chol	49.312	0.722	47.865	8.793
C18_TP_P	51.827	0.888	50.191	11.047
C18_TP_P_Chol	49.845	0.801	48.071	10.089

The overlay of DSC 1st heating runs of C16 based liposomal formulations is illustrated in Figure 7.1. The C16 empty liposomal formulation was used as a control.

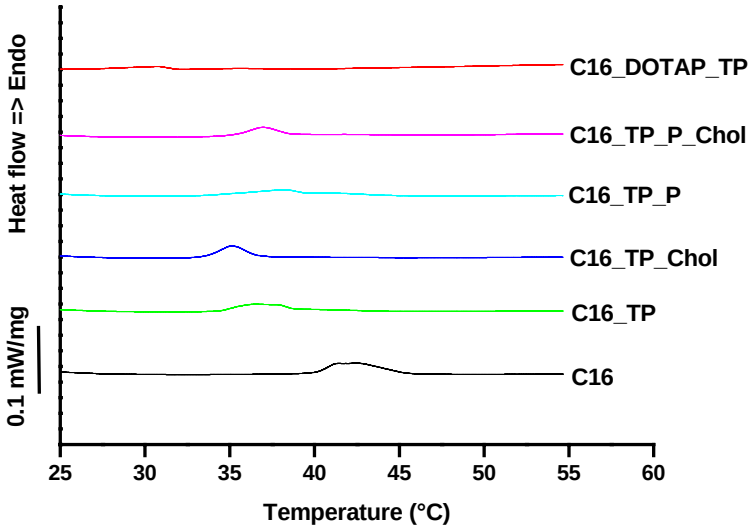

Figure 7.1: *Overlay of the DSC measurement of six donor C16 liposomal formulations with different compositions. All the peaks were from 1st heating run, at the heating rate of 5 °C / min. For a better comparison, the DSC curves were normalized to a sample weight of 1 mg and shifted along the ordinate in the figures.*

As indicated in Figure 7.1 and Table 7.2, empty C16 (DPPE/DPPG) liposomes have a main phase transition temperature T_m at 42.3°C. A small shoulder is noticed at a higher temperature of the phase transition peak. The presence of the shoulder can be probably explained as the high curvature radius of the liposome bilayer due to the reduced liposome size [Bonora S. et al., (2003); Castelli F. et al., (2005)]. The incorporation of temoporfin or further with PEG, Chol or DOTAP into C16 lipid membranes results in a distinct shifting of the phase transition temperature to lower temperatures and the decreased peak areas. The incorporation of TP (1.5 mg/ml, equal to 8.1 mol %) shifts the T_m to 36.5°C and the small shoulder still exists; the additional incorporation of Chol (0.9 mg/ml, equal to 8.5 mol %) gives rise to a further shift of T_m to 35.1°C and the presence of the shoulder is not any more pronounced. The addition of PEG (4.6 mg/ml, equal to 6.1 mol %) reduces T_m to 38.1°C, but with a much broader peak. Among them, the incorporation of DOTAP into C16 lipid membranes has the most significant influence of phase transition, with a reduced T_m at only 30 °C, and a much broader peak which is not easy to be detectable. As studied by Campbell R.B. and co-workers, the DSC measurements showed progressive decrease and broadening of the phase transition

temperature of DPPC with increasing fraction of DOTAP, in the range of 0.4-20 mol % [Campbell R.B., et al., (2001)].

The overlay of DSC 1st heating runs of C18 based liposomal formulations is illustrated in Figure 7.2. The C18 empty liposomal formulation was used as a control.

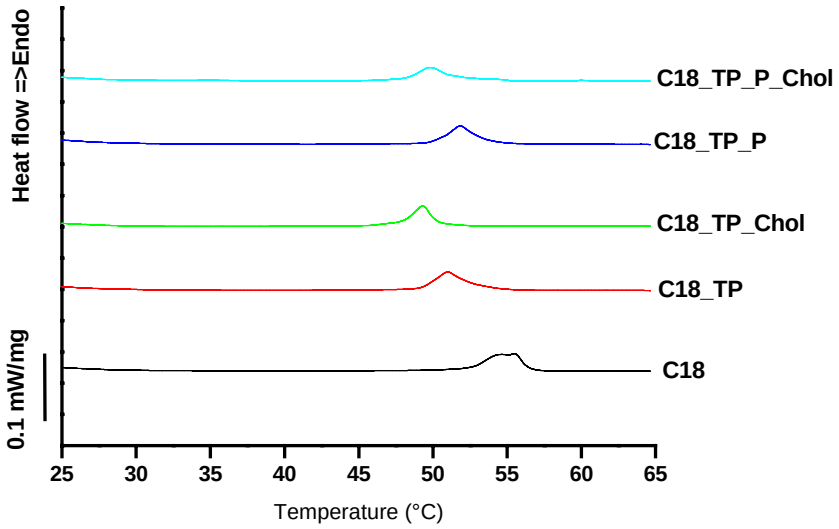

Figure 7.2: *Overlay of the DSC measurements of donor C18 liposomal formulations with different compositions. All the peaks were from 1st heating run, at the heating rate of 5 °C / min. For a better comparison, the DSC curves were normalized to a sample weight of 1 mg.*

As indicated in Figure 7.2 and Table 7.2, empty C18 (DSPC/DSPG) liposomes have a main phase transition temperature at 55.4°C and with the presence of a shoulder at the higher temperature. The values of the empty C16 and C18 liposomes are in agreement with previous studies [Cameron D.G. and Mantsch H.H., (1982); van Dijck P.W.M. et al, (1977); Small D.M., (1984)]. As demonstrated in the case of C16 liposomal formulations, the incorporation of temoporfin, or ,with PEG and Chol, into C18 liposome also results in the shifting of phase transition to lower temperatures. Furthermore, the peaks obtained are sharper and smaller than the peak in empty C18 liposomes. Similarly, the addition of TP into C18 lipid membranes results in a decrease of T_m to 51.0°C, and the additional incorporation of Chol gives rise to a further shift of T_m to 49.3°C. The addition of PEG reduces T_m to 51.8°C and does not produce a broadening of the peak as in C16 liposomes. As published in the previous study, the presence of cholesterol at a low concentration (< 10 mol %) in C14-C20 phosphatidylcholine bilayer, eliminates the phospholipid pre-transition, and produces a decrease in the T_m as the DSC endotherm broadens and the enthalpy of transition decreases [Malcomson R.J., et, al. (1997); McMullen R.P.W., et, al, (1993); Oldfield E. and Chapman D., (1972)]. Earlier

studies have also demonstrated that the main transition for DMPC and DPPC containing 13-25 mol % cholesterol is composed of two, or possibly three peaks, which could be explained as the coexistence of two immiscible solid phases [McMullen T.P.W. et al., (1993); Genz A. et al., (1986)]. In the work of Mabrey S., it is concluded that in DPPC multilamellar liposomes containing above 20 mol % cholesterol, only one broad peak is observed, and the peak disappears at about 50 mol % cholesterol [Mabrey S. et al., (1978)].

The temperature of 25 °C is usually selected as the physical model temperature and 37 °C is the normal body temperature. At 25 °C, the lipids of all the C16 and C18 TP containing liposomal formulations are at a rigid gel state. At 37 °C, the lipids of C16 TP containing formulations are in a liquid crystalline state, but the lipids of C18 TP containing liposomal formulations are still in the rigid gel state. The transfer of TP from different liposomal formulations at these two temperatures is studied.

7.3 Temoporfin transfer between liposomal membranes

The transfer kinetics of temoporfin from different donor liposomal formulations to acceptor at incubation temperatures of 25 °C and 37 °C was measured by fluorescence spectroscopy. The percentage transfer of TP from C16 TP containing liposomal formulations at different incubation time during 4.5 h at 25 °C is presented in Table 7.3.

Table 7.3: *Percentage transfer of temoporfin from four C16 donor formulations to acceptor liposomes during 4.5 h incubation at 25 °C. The results are obtained from the average of three repetitions.*

Incubation time (min)	C16_TP	C16_TP_P	C16_TP_Chol	C16_TP_P_Chol
1	0.69 ± 0.17	0.41 ± 0.66	3.80 ± 0.71	4.07 ± 1.69
5	2.37 ± 1.01	6.25 ± 2.41	8.07 ± 1.58	11.54 ± 2.24
10	4.08 ± 1.58	9.74 ± 1.57	16.63 ± 2.54	15.55 ± 2.64
15	5.33 ± 1.65	11.30 ± 1.88	22.30 ± 1.26	17.98 ± 1.58
30	10.34 ± 1.34	18.25 ± 1.26	31.45 ± 2.41	25.68 ± 1.47
45	14.29 ± 1.59	22.80 ± 2.41	36.32 ± 2.13	30.97 ± 2.22
60	17.35 ± 0.98	26.91 ± 2.01	38.88 ± 2.01	35.75 ± 0.91
75	19.43 ± 2.15	30.19 ± 1.04	41.07 ± 1.63	36.45 ± 1.60
90	21.94 ± 2.15	31.90 ± 1.65	42.69 ± 1.17	39.48 ± 1.42
105	22.88 ± 1.41	33.31 ± 1.58	43.55 ± 0.68	41.47 ± 1.74
120	25.07 ± 1.62	34.76 ± 2.16	44.71 ± 1.57	42.18 ± 1.36
150	28.03 ± 1.11	37.47 ± 0.95	46.68 ± 1.95	43.91 ± 1.57
180	28.91 ± 1.08	38.67 ± 1.43	47.84 ± 2.45	46.64 ± 2.14
210	29.81 ± 1.97	39.53 ± 1.24	48.77 ± 2.06	47.48 ± 2.24
240	30.25 ± 2.04	40.60 ± 1.75	49.34 ± 2.47	48.38 ± 1.57
270	31.06 ± 1.06	41.42 ± 2.14	50.21 ± 1.77	49.10 ± 1.63

The schematic graph of the overlay of TP transfer kinetics from four C16 donor formulations to acceptor liposomes in 10 mM Tris buffer at pH 7.4 during 4.5 h incubation at 25 °C is illustrated in Figure 7.3.

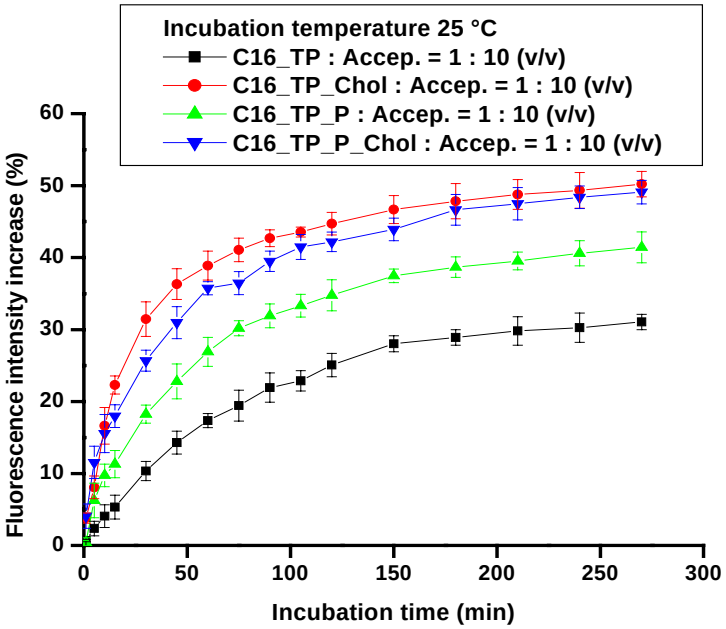

Figure 7.3: *Fluorescence percentage enhancement from four C16 donor formulations to acceptor during 4.5 hours incubation at 25 °C. The error bars are calculated from three repetitions.*

As shown in Figure 7.3 and Table 7.3, at 25 °C incubation temperature, the transfer of TP from different TP containing liposomal formulations shows different transfer kinetics. The conventional formulation C16_TP has the slowest transfer kinetics. The fastest transfer kinetics is obtained in C16_TP_Chol formulation, and then followed by the C16_TP_PEG_Chol and C16_TP_PEG formulations. After 270 min incubation, the fluorescence transfer level of cholesterol-containing formulations is distinctly higher than the conventional formulation and the PEG-containing formulations. Even though the phase transition temperatures of all the formulations are above the experimental temperature of 25 °C, an inversed proportion between phase transition temperature and the transfer speed is noticed among the three modified liposomal formulations. The phase transition temperature of the modified formulations follows: C16_TP_PEG > C16_TP_PEG_Chol > C16_TP_Chol, which is the opposite sequence of the transfer speed of these formulations.

The percentage transfer TP from C16 TP containing liposomal formulations at different incubation time during 4.5 h at 37 °C is presented in Table 7.4.

Table 7.4: *Percentage transfer of temoporfin from four C16 donor formulations to acceptor during 4.5 hours incubation at 37°C. The results are obtained from the average of three repetitions.*

Incubation time (min)	C16_TP	C16_TP_P	C16_TP_Chol	C16_TP_P_Chol
1	5.72 ± 1.24	3.56 ± 1.14	4.96 ± 1.46	10.96 ± 2.42
5	32.82 ± 2.12	29.93 ± 2.53	35.37 ± 3.14	30.85 ± 1.59
10	35.16 ± 1.68	32.64 ± 1.65	39.00 ± 2.12	34.07 ± 2.58
15	37.44 ± 2.14	34.04 ± 1.65	41.04 ± 1.65	35.49 ± 1.14
30	41.21 ± 1.69	37.78 ± 1.58	44.61 ± 1.46	39.53 ± 0.75
45	43.44 ± 1.46	39.49 ± 1.69	47.35 ± 2.25	41.49 ± 1.46
60	44.83 ± 2.89	40.62 ± 0.76	48.77 ± 1.54	42.44 ± 0.87
75	46.02 ± 1.21	41.84 ± 2.12	49.99 ± 1.87	43.56 ± 1.68
90	46.81 ± 2.54	42.40 ± 1.68	50.84 ± 1.01	43.71 ± 1.98
105	47.36 ± 1.87	42.94 ± 0.84	51.62 ± 0.95	44.16 ± 2.01
120	48.42 ± 2.24	43.48 ± 1.27	52.39 ± 2.42	44.71 ± 1.57
150	49.92 ± 1.54	44.64 ± 1.09	53.08 ± 1.62	45.81 ± 0.68
180	50.68 ± 1.46	45.29 ± 2.11	52.58 ± 1.75	45.68 ± 1.88
210	51.04 ± 1.14	45.66 ± 1.55	52.96 ± 1.41	45.71 ± 1.27
240	51.79 ± 2.12	45.88 ± 1.06	53.24 ± 1.62	45.83 ± 1.24
270	53.04 ± 1.19	46.39 ± 2.41	53.93 ± 0.14	45.67 ± 1.01

The schematic graph of the overlay of TP transfer kinetics from four C16 donor formulations to acceptor liposomes in 10 mM Tris buffer at pH 7.4 during 4.5 h incubation at 37 °C is illustrated in Figure 7.4.

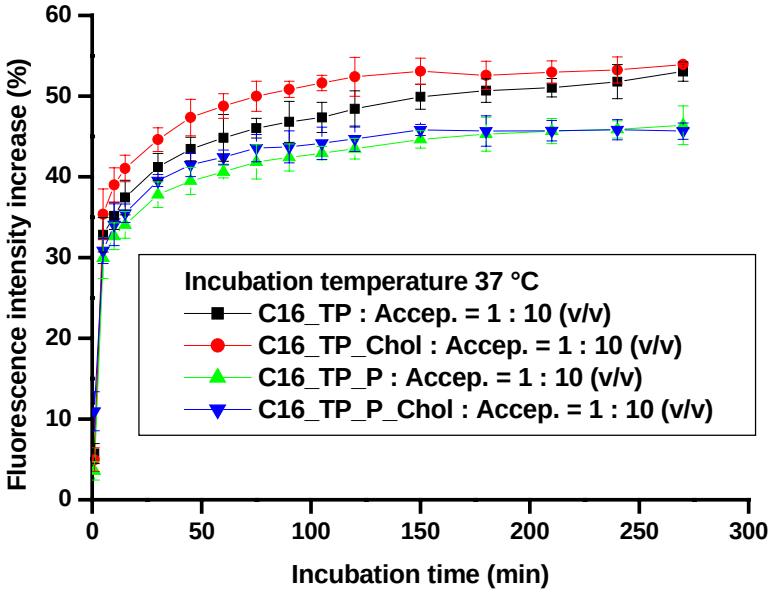

Figure 7.4: *Fluorescence percentage enhancement from four C16 donor formulations to acceptor during 4.5 hours incubation at 37 °C. The error bars are calculated from three repetitions.*

As indicated in Figure 7.4 and Table 7.4, at 37 °C incubation temperature, TP is transferred from all C16 liposomal formulations in a very fast manner. For the three modified liposomal formulations, the transfer kinetics is in the same sequence as in the one at 25°C. The transfer speed of conventional formulation increases dramatically to be faster than the two PEG-containing formulations, and almost reaches the same transfer speed as C16_TP_Chol formulation. It seems that after 150 min incubation, a stable plateau is obtained in the all the formulations including the conventional C16_TP formulation. The maximal TP transfer amount is within the range from 45 to 53 %. It can be noted that at 37 °C, the bilayer membranes of all the formulations are in a liquid crystalline state (the T_m of C16_TP_P is around 38 °C, which is quite close to 37 °C), so that the fluid membrane arrangement facilitates the transfer of the drug.

The percentage transfer TP from C18 TP containing liposomal formulations at different incubation times during 4.5 h at 25 °C is presented in Table 7.5.

Table 7.5: *Percentage transfer of temoporfin from four C18 donor formulations to acceptor during 4.5 hours incubation at 25°C. The results are obtained from the average of three repetitions.*

Incubation time (min)	C18_TP	C18_TP_P	C18_TP_Chol	C18_TP_P_Chol
1	0.66 ± 0.74	1.92 ± 0.09	2.37 ± 2.08	1.20 ± 1.42
5	0.79 ± 0.66	2.57 ± 0.22	5.33 ± 0.22	1.66 ± 0.22
10	1.05 ± 0.81	2.62 ± 0.08	7.35 ± 2.49	2.04 ± 1.42
15	1.25 ± 0.94	2.73 ± 0.29	7.89 ± 2.56	2.37 ± 1.12
30	1.59 ± 0.78	3.71 ± 0.40	10.68 ± 2.22	4.03 ± 1.76
45	2.01 ± 1.10	4.53 ± 0.40	12.37 ± 1.69	5.50 ± 2.63
60	2.50 ± 1.52	6.68 ± 1.47	13.99 ± 1.53	7.46 ± 3.10
75	2.98 ± 1.24	7.49 ±1.07	15.28 ± 1.86	9.23 ± 2.41
90	3.25 ± 1.15	8.01 ± 0.82	17.31 ± 2.18	11.67 ± 2.91
105	4.17 ± 0.87	9.23 ± 0.42	18.54 ± 1.42	13.51 ± 2.58
120	5.49 ± 0.57	10.17 ± 1.04	19.30 ± 1.38	15.41 ± 3.03
150	6.74 ± 0.64	12.36 ± 0.97	21.13 ± 1.83	18.87 ± 1.97
180	7.65 ± 1.58	14.32 ± 1.60	23.22 ± 2.03	21.46 ± 1.46
210	8.46 ± 1.54	16.48 ± 2.12	25.60 ± 2.42	23.44 ± 3.02
240	9.87 ± 0.86	17.79 ± 1.19	27.03 ± 2.43	28.80 ± 2.15
270	10.49 ± 0.47	19.01 ± 1.53	30.58 ± 1.47	31.54 ± 1.52

The schematic graph of the overlay of TP transfer kinetics from four C18 donor formulations to acceptor liposomes in 10 mM Tris buffer at pH 7.4 during 4.5 h incubation at 25 °C is illustrated in Figure 7.5.

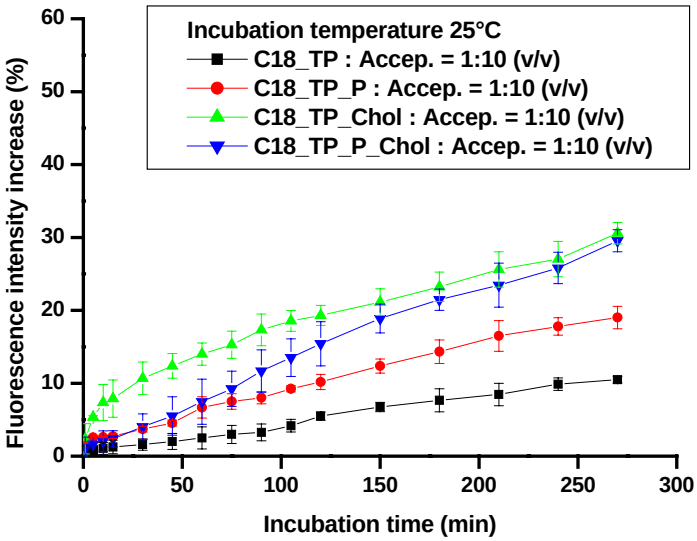

Figure 7.5: *Fluorescence percentage enhancement from four C18 donor formulations to acceptor during 4.5 hours incubation at 25 °C. The error bars are calculated from three repetitions.*

As indicated in Figure 7.5 and Table 7.5, the transfer of TP from all C18 liposomal formulations at 25 °C is much slower compared to the transfer from C16 liposomal formulations at the same incubation temperature. After 270 min incubation, the maximal transfer amount is 30 % from the formulation of C18_TP_Chol and C18_TP_P_Chol. Similar as the transfer behaviour of C16 (DPPC/DPPG) formulations, the conventional C18_TP formulation has the slowest transfer kinetics. The fastest transfer kinetics is obtained in C18_TP_Chol formulation. Except in the case of conventional formulation, for all the other modified formulations there is an increase of drug transfer kinetics with the decrease of phase transition temperature. The percentage transfer of TP from C18 TP-containing liposomal formulations at different incubation time during 4.5 h at 37°C is presented in Table 7.6 and the data of TP transfer kinetics from four C18 donor formulations to acceptor liposomes in 10 mM Tris buffer at pH 7.4 during 4.5 h incubation at 37°C represented in Figure 7.6.

Table 7.6: *Percentage transfer of temoporfin from four C18 donor formulations to acceptor during 4.5 hours incubation at 37°C. The results are obtained from the average of three repetitions.*

Incubation time (min)	C18_TP	C18_TP_P	C18_TP_Chol	C18_TP_P_Chol
1	2.29 ± 0.85	1.02 ± 1.17	2.18 ± 1.55	3.12 ± 2.53
5	7.55 ± 0.34	2.56 ± 0.76	5.1 ± 0.90	7.09 ± 3.14
10	11.19 ± 0.59	5.27 ± 0.66	11.25 ± 1.85	11.82 ± 1.95
15	13.54 ± 0.74	7.18 ± 0.86	16.48 ± 2.63	14.55 ± 2.62
30	20.12 ± 0.48	12.58 ± 0.43	30.91 ± 2.08	25.97 ± 3.55
45	25.30 ± 0.63	16.95 ± 1.18	40.30 ± 1.88	33.58 ± 3.40
60	30.34 ± 0.83	21.73 ± 1.45	46.15 ± 1.90	39.35 ± 2.10
75	34.48 ± 1.02	24.68 ± 1.48	50.84 ± 1.58	42.81 ± 2.01
90	37.66 ± 0.99	28.40 ± 2.27	56.29 ± 0.65	45.64 ± 2.05
105	39.84 ± 0.87	30.41 ± 1.68	57.46 ± 1.25	46.95 ± 1.95
120	42.36 ± 1.42	33.84 ± 1.55	60.97 ± 1.44	48.04 ± 1.99
150	45.55 ± 1.27	38.02 ± 0.74	63.98 ± 1.91	49.98 ± 1.43
180	47.73 ± 1.48	41.35 ± 1.99	65.29 ± 1.55	50.96 ± 1.34
210	49.93 ± 1.66	42.66 ± 1.01	66.65 ± 1.85	52.72 ± 1.05
240	51.16 ± 1.35	43.92 ± 1.02	67.38 ± 1.51	53.64 ± 1.22
270	52.03 ± 1.54	45.68 ± 1.53	68.41 ± 1.84	54.58 ± 1.56

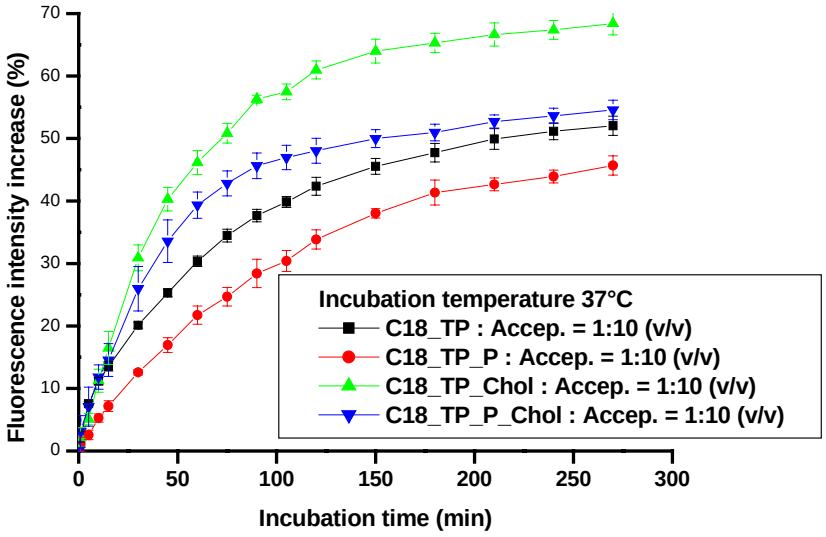

Figure 7.6: *Fluorescence percentage enhancement from four C18 donor formulations to acceptor during 4.5 hours incubation at 37 °C. The error bars are calculated from three repetitions.*

Figure 7.6 and Table 7.6 show that the transfer speed and transfer amount of TP from all C18 liposomal formulations during 270 min incubation are greatly increased at 37 °C compared to 25 °C, even though the lipids are still in a solid gel state at 37 °C. The slowest transfer kinetics is found in C18_TP_P formulation instead of the conventional C18_TP formulation

as in the C16 formulations. The C18_TP_Chol formulation has the fastest transfer speed, and with the highest transfer amount of 68 %.

Summary

All the donor C16 and C18 formulations prepared are large unilamellar liposomes with a size distribution around 110 nm, and PDI < 0.1. From the DSC study, the incorporation of cholesterol, PEG or temoporfin has the similar effect of shifting the phase transition temperature of the pure C16 (DPPC/DPPG) or C18 (DSPC/DSPG) liposomal formulation to a lower temperature, and with a decreased enthalpy and peak area. These alterations in the DSC results can be explained through that the lipophilicity of the incorporated substances leads to interaction with the acyl chains of the phospholipid [El Maghraby G.M.M. et al., (2005)].

The primary *in vitro* TP transfer experiments show very diverse transfer kinetics of TP from different liposomal formulations and at different temperatures. The transfer of TP from C16 liposomal formulations is faster than C18 liposomal formulations at both selected temperatures. Generally, the transfer of TP at a higher temperature is much faster than at a lower temperature. This phenomenon is more pronounced in the C16 liposomal formulations. The behaviour can be explained as follows: for C16 TP-containing liposomal formulations, the lipid bilayer membranes are in a gel state at 25 °C. At 37 °C the lipids change to a liquid crystalline state. The lipids in a solid gel state are more rigid compared to the lipids in a liquid crystalline state. Therefore, the release of temoporfin is confined at a lower temperature. For C18 TP-containing liposomal formulations, the temperature was below the phase transition temperature of the lipids at both 25 and 37 °C. However, the higher temperature could loosen in some degree the lipids arrangement, which could facilitate the release of temoporfin from donor to acceptor.

For C16 TP-containing liposomal formulations, the fastest transfer kinetics is obtained in C16_TP_Chol formulation at the two selected temperatures. A similar behaviour is observed in C18_TP-containing liposomal formulations. This phenomenon could be explained as follows: the lipid membrane with the incorporation of cholesterol has the lowest phase transition temperature. It is known that the lipids with a lower phase transition temperature have higher lipid membrane fluidity, thus the drug release from the lipid membrane with a higher fluidity is much easier than from a rigid lipid membrane. Even though cholesterol is also known to regulate the membrane, it produces a condensing effect on membranes and make them more rigid [López-Pinto J.M., et al., (2005)]. This membrane-regulation effect is apparently not the dominant factor of influencing the transfer of temoporfin compared to the phase transition temperature reduction effect. As a result of the competition of these two

effects, the transfer of TP from cholesterol-containing formulation demonstrates the fastest transfer kinetics at both of the tested temperatures.

The incorporation of PEG or into lipid bilayer exhibits faster transfer kinetics than the conventional formulations only at the lower temperature. At the higher temperature, this superiority is overcome by the conventional C16_TP formulation. A similar behaviour is observed in C18_TP-containing liposomal formulations. PEG is known to increase the lipid fluidity, which would facilitate the drug transfer. But the big head group of PEG could also prevent the collision of donor with acceptor liposomes, which in contrary will inhibit the drug transfer. At a lower temperature, the membrane arrangement of PEG-containing liposomes is in a much more fluid state compared to the conventional C16_TP or C18_TP, therefore the drug in PEG-containing liposomes could be much easier released than the conventional formulation. At a higher temperature, the lipids bilayer membrane in C16 formulations is already in a fluid crystalline state, and in C18 formulations is also in a more fluid state than at the lower temperature. Therefore, the transfer of TP can be easily released from both the conventional and modified liposomal formulations. This is demonstrated through a much faster TP transfer of all the formulations at a higher temperature. But due to the big head group of PEG, the collision contact of PEG-containing donor liposomes with acceptor liposomes is, to a certain extent, decreased compared to the conventional formulations with the acceptor liposomes. Another possible explanation for this phenomenon at a higher temperature is that, some of the temoporfin will transfer directly into the big head groups of PEG, instead of acceptor liposomes. As a result, the transfer rate is slower and the transfer amount is lower of TP from PEG modified formulations compared to the conventional formulations.

Finally, it is worth to mention that at the higher temperature, the transfer of TP tends to reach a plateau after 150 min incubation. The maximal transfer of TP from C16 liposomal formulations to acceptor liposomes is around 53 %, and a value of 68 % is obtained in C18_TP_Chol formulation. Theoretically, if there is the same partition coefficient of TP between donor and acceptor liposomes, a total amount of 99 % TP should be transferred into acceptor liposomes. This is obviously not the case in the experiments performed. DPPC and DSPC are saturated lipids, but the POPC used in acceptor liposomes is unsaturated. Thus, the differences in lipid character might be the reason of the less transfer amount of TP.

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Chapter 8

Conclusions

In the present work, the mechanisms and factors that influence the transfer kinetics of paclitaxel, cholesterol and cholesteryl-oleoyl-ether (COE) between the liposomal membranes has been investigated through drug transfer based on ion-exchange micro-column technique. The experiments were performed by following the transfer of the compound from charged, unilamellar 1,2-Dioleoyl-sn-Glycero-3-phosphocholine (DOPC) donor vesicles to neutral 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) acceptor vesicles. Vesicles were incubated in the absence of proteins and were stable to fusion over the course of the experiments. The charged drug-containing donor liposomes and neutral acceptor liposomes were incubated at room temperature, with the acceptor liposomes five-time in excess to donor liposomes. At selected time points during the incubation, a certain amount of the incubation mixture was applied on the columns, and eluted with 1.5 ml water. The drug transfer from donor to acceptor liposomes at each time point was measured by HPLC (for paclitaxel transfer) or LSC (for cholesterol and COE transfer).

For the drug transfer based on fluorescence dequenching technique, the transfer (or the release) of temoporfin from donor liposomes can be estimated by the increase of fluorescence intensity at different time points during the incubation of donor and acceptor liposomes. Temoporfin shows an evident self-quenching effect at quite low concentration, and the release of temoporfin gives rise to enhanced fluorescence intensity. This character is proved to be the easiest and fastest method of demonstrating the transfer kinetics of temoporfin from donor to acceptor liposomes. Eight different temoporfin-containing donor liposomal formulations were

prepared, in order to investigate the influence of cholesterol, PEG, and lipid types on the transfer of temoporfin.

8.1. Transfer kinetics of paclitaxel, cholesterol and cholesterol derivative

In a simple model system which is not influenced by fusion or the presence of proteins, two models can be considered to explain the mechanism of the transfer behaviour of the lipophilic compounds. These two models are named as the "Collision complex model" and the "Aqueous diffusion model". During the drug transfer process, both of these two mechanisms can influence the transfer kinetics. In the "Collision complex model", either diffusion within the collision complex or collision frequency may be rate limiting and, also, collisions are blind to the compound transferred. On the other hand, collisions between lipid molecules (presumed to be present in the water as monomers) and the acceptor vesicle in the adsorption step would proceed at a rate proportional to the concentration of acceptor vesicles. In the "Aqueous diffusion model", the desorption of the compound molecules from the donor bilayer into the aqueous phase is the rate limiting step. Aqueous diffusion may follow either first- or second-order kinetics depending on the rate-limiting step in the exchange. First-order kinetics is predicted when desorption is the rate-limiting step. During the drug transfer process, one mechanism can have a stronger influence than another, but it is quite difficult to completely exclude one mechanism from another. Based on the understanding of these two transfer mechanism models, the rate of transfer of a "Collision complex model" dominated transfer should be much faster compared to an "Aqueous diffusion model" dominated transfer. In the "Collision complex model", the time-consuming step of desorption of the compound into aqueous phase can be neglected. Therefore, the nearly instantaneous transfer of paclitaxel might indicate a preference for the "Collision complex model" than for the "Aqueous diffusion model". The incorporation of PEG decreases only the transfer amount of paclitaxel, but not the transfer speed. Some amount of paclitaxel might transfer into the big head groups of PEG instead of the bilayer of acceptor liposomes.

The possible mechanisms of cholesterol transfer have been investigated by many researchers, but no complete agreement has been reached. In this study, the transfer of cholesterol from both positively and negatively charged donor liposomes was investigated. The transfer of cholesterol from these two types of donor liposomes showed quite surprising diversity in the transfer kinetics. The transfer of cholesterol from negatively charged donor liposomes followed a perfect exponential kinetics, with a time constant at around 58 minutes. This value is quite comparable with the discovery in a previous work by Fahr A. and co-workers. It is

worth to mention that, the transfer of cholesterol from negatively charged donor liposomes was performed under nearly the same conditions as in the work of Fahr A. and co-workers, with the only difference in donor acceptor incubation ratio. In the present study, an incubation ratio of 1 to 5 was selected instead of 1 to 10 as in the work of Fahr A. and co-workers. From the obtained results, it can be observed that the difference in acceptor amount does not give rise to a proportional change in cholesterol transfer rate. The transfer of cholesterol from negatively charged donor liposomes seems to be dominated by the "Aqueous diffusion model" instead of "Collision complex model". The transfer of cholesterol from positively charged donor liposomes showed, however, a two-step transfer process, i.e., one fast transfer process followed by a slow transfer process. The occurrence of the fast transfer process could be attributed to the incorporation of DOTAP into the lipid bilayer. DOTAP gives rise to a deteriorated retention of cholesterol in the positively charged donor liposomes. The unsaturated acyl chains and the charged big head groups of DOTAP are supposed to influence in a high degree the lipid membrane arrangement and fluidity. In presence of DOTAP, the molecular interaction of cholesterol with lipid bilayer is decreased. As a result, cholesterol is released more easily from the positively charged donor liposomes. However, DOTAP could only facilitate part of cholesterol transfer. Once more than 50 % cholesterol is transferred into acceptor, the transfer speed of cholesterol from positively charged donor liposomes decreased noticeably to a similar rate as the transfer speed of cholesterol from negatively charged donor liposomes. Thus, the transfer of cholesterol from positively charged donor liposomes might follow a similar transfer mechanism as the transfer of cholesterol from negatively charged donor liposomes, but the transfer rate is partially accelerated by the incorporation of DOTAP.

The transfer of cholesterol derivative, cholesteryl-oleoyl-ether (COE), is considered as non-exchangeable between the liposomal membranes, in the case of both positively and negatively charged donor liposomal formulations. As indicated by the transfer kinetics, the COE molecule should have a strong affinity with the lipid membranes.

The calculated log P values of paclitaxel and cholesterol are around 4.73 and 9.52, as calculated by Bio-Loom Windows Software Version 1.0. The log P value of COE is not available. These values are only a theoretical value of the compounds distribution coefficient between octanol and water. The log P value alone could not predict the transfer behaviour of the compounds between the liposomal membranes. There are more factors which could influence the transfer of compound between liposomal membranes: the molecular structure of the compound, such as the functional groups and the interaction with the lipid molecular; and also the lipid types, i.e., the lipid chain unsaturation, lipid chain length or headgroup types,

etc. Moreover, the incorporation of other substances, such as cholesterol, PEG or DOTAP, will also influence the compound transfer behaviour.

8.2. Transfer kinetics of temoporfin

It has been found in the temoporfin transfer study that the carbon chain length and saturation status of the phospholipid, the incorporation of cholesterol, 1,2-Dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DPPE-mPEG2000) or 1,2-Distearyl-phosphatidylethanolamine-methyl-polyethyleneglycol-2000 (DSPE-mPEG2000) and the incubation temperature have an influence on the transfer of temoporfin. Under the conditions used in the experiment, several conclusions could be drawn:

i) A higher incubation temperature accelerates the transfer of temoporfin. The lipid has higher membrane fluidity at a higher temperature, therefore, the incorporated drug can be released easily from the lipid membranes with higher fluidity. For temoporfin-containing DPPC/DPPE liposomal formulations, the lipid is at a liquid crystalline state at 37 °C, and the lipid is at a solid gel state at 25 °C. The loose lipid arrangement at a liquid crystalline state facilitates the transfer of temoporfin. Moreover, the molecular movement is much faster at a higher temperature than at a lower temperature. That is why even though the incubation temperature of 37 °C is still below the lipid phase transition temperature of temoporfin-containing DSPC/DSPE liposomal formulations, the transfer rate of temoporfin is greatly increased at 37 °C compared to the incubation at 25 °C.

ii) The longer the lipid chain length the slower the temoporfin transfer rate. The phase transition temperature of the saturated lipid increases with the lipid chain length. The lipid with a higher phase transition temperature has more rigid lipid membrane arrangement, which is not easy for the transfer of temoporfin.

iii) The incorporation of cholesterol facilitates the transfer of temoporfin in both DPPC/DPPE and DSPC/DSPE lipid based formulations. On the one hand, cholesterol has the ability of regulating the lipid membrane arrangement and make the membrane more rigid, which has the effect of decreasing the transfer rate of temoporfin. On the other hand, the incorporation of cholesterol decreases at the same time the phase transition temperature of the lipid, which is helpful for the transfer of temoporfin. Under the influence of these two effects, the incorporation of cholesterol gives rise to an increased temoporfin transfer.

iv) The incorporation of PEG facilitates the transfer of temoporfin more effectively at a lower temperature than at a higher temperature when comparing the transfer behaviour of temoporfin from conventional liposomal formulation (C16_TP or C18_TP) and PEG-

modified liposomal formulation (C16_TP_PEG or C18_TP_PEG). PEG is known to increase the lipid fluidity, which would facilitate the drug transfer. But the big head group of PEG could also prevent the collision of donor with acceptor liposomes, which oppositely will inhibit the drug transfer. It is also important to consider that, with the incorporation of PEG, some of temoporfin will transfer to PEG groups instead of acceptor liposomes. At a lower temperature, the membrane fluidization effect of PEG plays a more important role than the prevention effect of PEG head groups. Therefore, the drug in PEG containing liposomes could be much easier released than the conventional formulation. At a higher temperature, due to the faster movement of the drug molecular, more temoporfin moves to PEG head groups instead of the acceptor liposomes. Therefore, the transfer of temoporfin from PEG-modified liposomal formulations are smaller than the conventional liposomal formulations.

In this study, the concentration of cholesterol or PEG in the lipid is fixed. It is not known that the difference in concentration of cholesterol and PEG has different influences on the drug transfer. A maximal temoporfin transfer value of 99 % is expected since the incubation ratio of donor and acceptor liposomes is 1 to 100. Here, the highest transfer amount of temoporfine from all the formulations obtained is around 50 %. This is much lower than the theoretical transfer value. Temoporfin might have a different partition coefficient between the donor and acceptor liposomes and more affinity to the host lipid membranes.

Appendix 1

Linear relation of PXL transfer from Donor (PXL) into Acceptor (0-50 mM) liposomes

As reported in the literature, the water solubility of PXL is found to be 0.50 ± 0.05 µM [Wenk M.R. et al., (1996)]. This means that there is always the equilibrium of 0.5 µM PXL dissolved in water. Under this conditions, and together with the assumption that PXL is equally distributed between donor and acceptor liposomes, the transfer of PXL from donor PXL-containing liposomes should be proportionally linear to the ratio of [Acceptor]/([Donor] + [Acceptor]). Based on these assumptions, the linearity experiment is designed.

Donor (PXL) and donor (PXL-PEG) liposomes are prepared with the lipid composition as: DOTAP/DOPC/DOPE-Rho/PXL=25/71/1/3, molar ratio, and DOTAP/DOPC/DOPE-Rho/DOPE-PEG/PXL=25/66/1/5/3, molar ratio, respectively. The lipid concentration of both liposomal formulations is 10 mM. Donor liposomes is incubated with equal volume of acceptor liposomes, 50 µl from the incubation suspensions were finally diluted with 1.5 ml water. So that the total amount of PXL in the eluent should be 5 µM. The theoretical values of the transfer of PXL from donor to acceptor liposomes at different acceptor lipid concentrations are calculated by assuming a total PXL amount of 5 µM.

During the linear PXL transfer experiment, it is observed that a constant amount of 1 µM of PXL is recovered in the eluent when donor liposomes is incubated with water, instead of the acceptor liposomes (See Table A-1, the PXL concentration at [Acc] equal to 0). This 1 µM of PXL might be from two sources: the free PXL dissolved in water at a concentration of 0.5 µM, and the PXL incorporated into donor liposomes, which also have the concentration of 0.5 µM. This is the part of donor (PXL) liposomes which could not be captured by the ion-exchange micro-columns and recovered together with acceptor liposomes in the eluent. This 1 µM of PXL recovered stably in the eluent is subtracted from all the experimental results obtained.

As measured by the radioactivity in a parallel experiment, the acceptor recovery is in the range from 87 to 92 % (See Table 6.13). The value of 89 % is decided to be the constant acceptor recovery in all the incubations. Therefore, the obtained experimental values of PXL transferred into acceptor liposomes in the eluent should be normalized to 100 %. The 1 µM of PXL, which presented constant in the eluent should not be involved in this normalization.

Based on these considerations, the experimental data are further normalized and the detailed calculation is demonstrated in Table A-1.

Table A-1. *The detailed calculation of the theoretical values (indicated as $[PXL]_{Th}$), the original experimental values (indicated as $[PXL]_{Ex}$) and experimental values after subtracting 1µM (indicated as $[PXL]_{Acc}$) and the experimental values after normalization (indicated as $[PXL]_{Acc}$ norm) in the incubation of Donor (PXL) with Acceptor (0-50 mM) liposomes experiments. Three repetitions were performed at each acceptor lipid concentration.*

[Acc.] (mM)	[Acc]/([Don] + [Acc])	$[PXL]_{Th}$ (µM)	$[PXL]_{Ex}$ (µM)	$[PXL]_{Acc}$ (µM)	$[PXL]_{Acc}$ norm (µM)
0	0	0	1.01 ± 0.11	--	--
10	0.5	2.00	2.00 ± 0.13	1.00	1.12 ± 0.13
20	0.67	2.80	2.30 ± 0.28	1.30	1.46 ± 0.28
30	0.75	3.25	2.85 ± 0.42	1.85	2.08 ± 0.42
40	0.8	3.50	2.74 ± 0.30	1.74	1.96 ± 0.30
50	0.83	3.67	3.13 ± 0.58	2.13	2.39 ± 0.58

Appendix 2

Linear relation of PXL transfer from Donor (PXL-PEG) into Acceptor (0-50 mM) liposomes

The calculation of PXL transfer from donor (PXL-PEG) formulation to acceptor liposomes is executed in the same way as for donor (PXL) formulation, except the amount of PXL that should be subtracted. In the case of donor (PXL-PEG) formulation, due to the inadequate donor (PXL-PEG) zeta-potential, the separation efficiency of the ion-exchange columns is greatly decreased. The amount of PXL recovered in the eluent in donor blank experiment increased to 2 µM (See Table A-2, the PXL concentration at [Acc] equal to 0). Therefore, the amount of 2 µM of PXL is subtracted from all the experimental values obtained. The detailed calculations of the normalization of the experimental data are shown in Table A-2.

Table A-2. *The detailed calculation of the theoretical values (indicated as $[PXL]_{Th}$), the original experimental values (indicated as $[PXL]_{Ex}$) and experimental values after subtracting 1µM (indicated as $[PXL]_{Acc}$) and the experimental values after normalization (indicated as $[PXL]_{Acc}$ norm) in the incubation of Donor (PXL-PEG) with Acceptor (0-50 mM) liposomes experiments. Three repetitions were performed at each acceptor lipid concentration.*

[Acc.] (mM)	[Acc]/([Don] + [Acc])	$[PXL]_{Th}$ (µM)	$[PXL]_{Ex}$ (µM)	$[PXL]_{Acc}$ (µM)	$[PXL]_{Acc}$ norm (µM)
0	0	0	2.00 ± 0.09	--	--
10	0.5	2.00	2.60 ± 0.05	0.60	0.67 ± 0.05
20	0.67	2.80	3.66 ± 0.24	1.66	1.87 ± 0.24
30	0.75	3.25	3.88 ± 0.96	1.88	2.11 ± 0.96
40	0.8	3.50	3.42 ± 0.22	1.42	1.60 ± 0.22
50	0.83	3.67	3.41 ± 0.57	1.41	1.58 ± 0.57

Selbstständigkeitserklärung:

Hiermit erkläre ich Xiaoyi Zhu, dass ich die vorliegende Arbeit selbstständig und nur unter Verwendung der angegebenen Hilfsmittel und Literatur angefertigt habe.

Xiaoyi Zhu

Jena, den 26.09.2008

Curriculum Vitae ZHU XIAOYI

Personal data:

Name:	ZHU, Xiaoyi
Date of birth:	28-04-1982
Martial status:	Single
Nationality:	P.R. China
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Present position:

Ph.D. student at the Department of Pharmaceutical Technology of the Friedrich Schiller University of Jena, Germany.

Topic: **The transfer of different lipophilic drugs between liposomal membranes by using two *in vitro* models.**

Education:

- ***Master in Pharmaceutical Sciences*** from department of Pharmaceutical chemistry and drug analysis, Catholic University of Leuven, Belgium (Sep 2004– Oct 2005).

  Overall result: *magna cum laude* – 80.8 %

  Project: **Extractability of 2,4-dichlorobenzoic acid from oral dispensers.**
- ***Bachelor of Pharmacy*** from Faculty of Pharmacy, Fudan University, Shanghai, P. R. China (2000 – 2004).

  Project: **Panax notoginseng intranasal powders: effects on cardio-cerebrovascular diseases study.**

Teaching experience:

Supervision of students at pharmaceutical practical courses in German language

Pharmaceutical technology: 3rd and 7th semester, 5.2 h/week

Other experience:

- Intern in Pharmacy Faculty of Huashan Hospital, Shanghai, 2003.

- Intern in Johnson & Johnson Company, Shanghai Branch for non-steroid drugs researches, 2002.

Laboratory skills:

- Development and validation of analytical methods using **liquid chromatography with UV detector (HPLC-UV)**.
- Analysis of drugs and pharmaceuticals according to monographs of European Pharmacopoeia, especially using **HPLC-UV**.
- **Drug transdermal permeation** studies.
- Validation of analytical instruments.
- Preparation of different formulations like liposome, gel, emulsion and suppository.
- Researches in pharmacokinetics and pharmacodynamics of different formulations like liposome, intranasal gel and intranasal powder.
- Phase transition behaviour of liposomes by using **Differential Scanning Calorimetry (DSC)**.
- Drug transfer kinetics from liposomal preparations by using **Fluorescence Spectrometry**.
- Cell culture and cell binding experiments via flow cytometry of fluorimetry.

Language skills:

Chinese: native

German: fluent (TestDaF: 16/20)

English: excellent (TOEFL: 597/650; GRE: 1230/1400)

Spanish: basic level (learning)

Publications

Zhu X., Liu X., Fahr A., Transfer of PXL and cholesterol between liposomal membranes by using the ion-exchange micro-column model, (in preparation).

Conference contributions:

- Controlled Release Society (CRS) German Chapter Annual Meeting, Jena, February 23-24, 2006
- 18. Mountain / Sea Liposome Workshop in Ameland (Netherlands), September 18– 22, 2006
  Oral Presentation: **The investigation of transfer of lipophilic drugs in liposomes**
- Liposome Seminar in Meiningen (Germany), November 08-10, 2006
  Oral Presentation: **Pharmacokinetic studies of liposomal formulations of temoporfin**